# The transcription regulator DC-SCRIPT in dendritic cell and cancer biology

***Multiple faces of a single factor***

**Saartje Hontelez**

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# Chapter 1

## General introduction

## THE IMMUNE SYSTEM

Throughout natural history, organisms have developed different defense strategies to protect them from colonization by invasive microbes and viruses. These are generally referred to as immune responses and are part of the immune system of an organism. Even simple, unicellular life forms possess these protection mechanisms, usually involving antimicrobial peptides and enzyme systems to fight off potential pathogens. More complex, multicellular organisms, however, are more vulnerable to infections due to increased body mass and lifespan. Hence more sophisticated mechanisms developed, creating a layered defense system with increasing levels of specificity.

The best studied and most refined immune system can be found in Gnasthostomata, or jawed vertebrates, which includes humans. In these animals three layers of defense can be discerned. The first layer entails the epithelial and mucosal barriers, which prevent access of infections agents to the host's body. Pathogens that have breached this barrier encounter the secondary line of defense, the innate immune response. The cells and molecules of the innate system are able to clear the infection, or hold it in check until the third line of defense, the adaptive immune response, is activated. Adaptive immunity is the most specific defense, tailoring effector mechanisms to most efficiently clear the present pathogens. Importantly, the adaptive system generates immunological memory, preventing reinfection of the same, or similar, pathogens.

Microbes can, however, also engage mutualistic interactions with their host, for example in the gut where they aid digestion and prevent colonization of pathogenic bacteria. This requires the host to be tolerant for the presence of these microbes, and avert their eradication by the immune system. Furthermore, responses directed to harmless compounds, or healthy host cells and tissues should also be prevented, in order to avoid development of allergies or auto-immune diseases, respectively. In contrast, host cells that have been infected by viruses or transformed into tumor cells should be detected and cleared by the immune system. Tight control of both the innate and the adaptive arm of the immune system is therefore essential for survival of the host.

### *Innate immunity*

Discriminating dangerous pathogens and transformed host cells from harmless microbes and healthy tissue is the first task of the innate immune system. This requires recognition and uptake of microbes and apoptotic cells, which is facilitated by phagocytes such as neutrophils, macrophages and dendritic cells (DCs).[1] Pathogen recognition is essentially achieved through detection of non-self molecules, termed Pathogen Associated Molecular Patterns (PAMPs).[2] These structures are conserved among microbial species, and are essential for their survival and pathogenicity. Immune escape through mutation or deletion of these molecules will therefore impair the microbes' ability to infect and colonize the host.

Detection of PAMPs by cells of the innate immune system is facilitated by specialized, germ-line encoded receptors termed Pattern Recognition Receptors (PRRs). Currently, four classes of PRRs are recognized. The transmembrane Toll-Like Receptors (TLRs) and C-type lectin receptors (CLRs) are specialized in detection of exogenous- or phagocytosed pathogens, whereas the cytoplasmic Retinoic acid-inducible gene (RIG)-I-like receptors (RLRs, also known as RIG-I helicases (RLHs)) and the NOD-like receptors (NLRs) recognize intracellular microbes and viruses.[2,3] Besides PRRs, also Scavenger Receptors, Integrins and Fc Receptors have been shown to be involved in pathogen detection (for more detail on PRRs and PAMPs see: Chapter 2).

PRR expression differs among different cell types. Receptors detecting extracellular microbes or infected host cells are most commonly found on specialized immune cells, whereas PRRs recognizing intracellular pathogens are more broadly expressed. Viruses and other intracellular pathogens can potentially infect all host cells, causing activation of the intracellular NLRs[4] and RLRs[5]. Intracellular signaling induced by these PRRs results in production of Interferons (IFNs) that attract and activate innate immune cells, and induce resistance to viral replication.[6]

Pathogen recognition by the innate immune system is generally considered to be unspecific, discriminating only between broad classes of microbes. However, recent insights suggest that PRR cross-talk enables the innate system to discern between different microbial species, and tailor the response accordingly (described in: Chapter 2). For example, the response to the pathogenic yeast *Candida albicans* was shown to be much stronger than to the nonpathogenic yeast *Saccharomyces cerevisiae*, due to different PRR combinations involved in recognition of both species.[7] In addition, next to pathogen recognition, PRRs have also been suggested to bind host-derived molecules released from damaged cells, termed Damage Associated Molecular Patterns (DAMPs).[3] This importantly triggers tissue reparative responses, however, in some cases can also evoke immune activation against host cells and tissues, causing auto-immunity.[6]

Essentially two important innate effector mechanisms can be induced upon pathogen detection: (i) the cellular response and (ii) activation of the complement system. Innate cellular responses are elicited by neutrophils, macrophages and DCs, which induce production of reactive oxygen species (ROS) upon microbe phagocytosis, and degrade the ingested microbe.[8] In addition, pathogen recognition by these cells activates intracellular signaling pathways, resulting in the secretion of cytokines and chemokines. These molecules cause infiltration of more innate immune cells to the infected area, thereby reinforcing and sustaining the innate immune response.[9] Furthermore, release of cytokines and chemokines activates natural killer (NK) cells, which play an important role in the eradication of virus infected cells and tumor cells.[10]

Pathogen infection may also trigger activation of the complement system, a group of plasma proteins that can bind the cell surface of microbes, but not of host cells. Upon activation, the complement system coats the pathogen with complement proteins, aiding phagocytosis. However, complement binding can also directly destroy microbes through a cascade of proteolytic reactions that cause lesions in the pathogen's membrane.[11]

Finally, activation of the innate system can trigger, direct and regulate adaptive responses. This is essential, since most infections cannot be cleared solely by the innate system. In addition, the adaptive system further specifies the response and creates immunological memory.

*Adaptive immunity*

For proper and specific responses the adaptive system needs to be informed on the pathogenic target. This happens through presentation of pathogen derived antigens, and is facilitated by so called Antigen Presenting Cells (APCs). These cells encompass the innate macrophages and DCs, as well as the adaptive B lymphocytes (B-cells).[12]

Antigen presentation occurs via two important molecules termed Major Histocompatebility Complex (MHC) I and II. Classically, MHC-I molecules present endogenous proteins, whereas MHC class II display exogenous proteins. Virtually all nucleated cells are capable of presenting endogenous self- and viral peptides via MHC class I presentation, however only APCs can also present exogenous peptides using MHC class II molecules.[12-14] Besides MHC, also the major histocompatibility complex class I related chain (MIC) A and B have been identified as antigen presenting molecules. They are expressed by epithelial cells, fibroblasts, monocytes, dendritic cells and endothelial cells, in response to stress. MIC do not present peptides to T cells via interaction with the T-cell receptor (TCR), but instead bind the NKG2D receptor on NK cells and some T cells.[15-17]

Presentation of antigens by APCs activates the two effector arms of the adaptive immune system: (i) the cellular response and (ii) the humoral response. Importantly, activation occurs only when antigen presentation is accompanied by high expression levels of co-stimulatory molecules (figure 1). Co-stimulation is facilitated by the cell surface receptors CD40, B7.1 (CD80) and B7.2 (CD86), and the secretion of pro-inflammatory cytokines.[18] Expression of these molecules only occurs when captured antigens are accompanied by PAMPs that activate PRRs, thus preventing responses to presented self antigens. Indeed, immunodominant antigens generally also have PAMP activity, ensuring that the antigen is of microbial origin.[6]

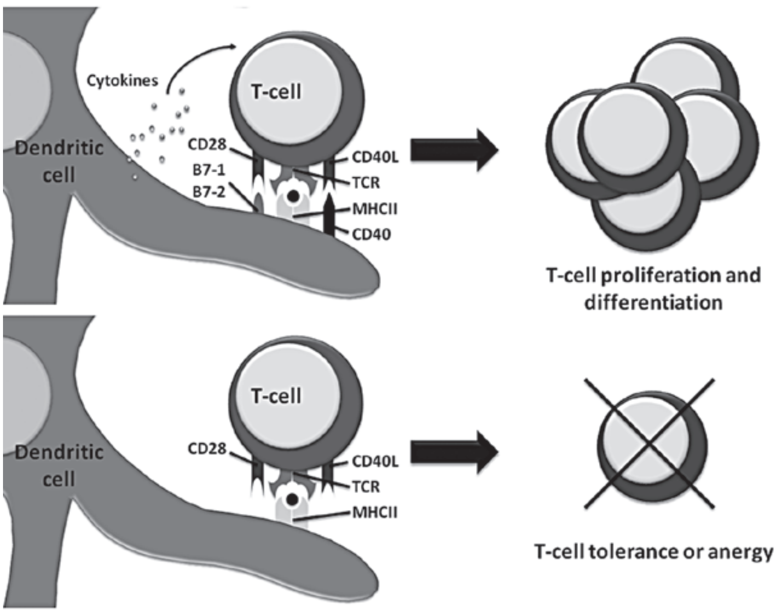


***Figure 1. Stimulation of T-cells by DCs.*** *Dendritic cells present antigens to T-cells in the presence of co-stimulatory molecules such as B7-1 and -2, CD-40 and secretion of cytokines. High levels of co-stimulatory signals results in T-cell activation and proliferation, whereas low or inhibitory co-stimulatory signals induces T-cell tolerance or anergy.*

The cellular response of the adaptive immune system involves activation and proliferation of two types of T lymphocytes (T-cells), the $CD8^+$ cytotoxic T-cells (CTLs) and the $CD4^+$ T-helper cells (Th). T-cells express a receptor (T-cell receptor, or TCR) that specifically binds the MHC molecule in combination with its presented antigen. Each T-cell expresses a unique TCR that originates from rearrangement of antigen receptor genes during T-cell development. Activated CTLs directly recognize and kill infected and/or transformed host cells upon activation.[19] Th-cells on the other hand, are important in providing ‘help’ to other cells of the immune system via direct interaction and cytokine production.[20]

One important cell type that receives help from Th-cells are the B-cells. These cell represent the humoral arm of the adaptive immune system, which is very effective in eradication of extracellular pathogens. B-cell activation and the generation of antibody-producing cells requires sequential phases. Initially, B-cells are activated upon capturing antigens with their specialized B-cell receptor (BCR). As with the TCR, also the BCR differs among B-cells, with each B-cell expressing a unique BCR. B-cells process and present the endocytosed antigens on MHC-II molecules, and migrate to the T-cell zones. Here, B-cells interact with activated Th-cells, which triggers B-cell proliferation and differentiation. Finally, interaction with antigen presenting follicular DCs (FDCs) in the germinal centers of the lymph nodes prevents apoptosis of the activated B-cells. The surviving B-cells differentiate into plasma cells, producing high amounts of antibodies that bind specific microbial antigens. Antibody binding prevents infections by intracellular microbes, and aids their elimination through facilitating phagocytosis and complement activation.[21]

Both T- and B-cell activation generates long-lived memory cells, which are much more efficient in generating immune responses upon reinfection of the same or similar pathogens, compared to naïve T- and B-cells.[21,22]

## DENDRITIC CELLS

Dendritic cells are the most efficient APCs of the immune system, and importantly function as a bridge between innate and adaptive immunity. The first observation of DCs dates back to 1868, when the medical student Paul Langerhans discovered a new cell population in the human skin. These cells were termed 'Langerhans Cells' (LC). However, due to their dendritic nature and typical 'neuronal' gold chloride stain, he mistakenly characterized them as nerve cells.[23] It was not until 1973 that Steinman and Cohn first used the term 'dendritic cell', and identified DCs as part of our immune system.[24] DC characterization has been ongoing ever since, increasing our understanding on the origin and function of these cells. In time, DCs have been increasingly appreciated for their central role in the immune system, initiating and modulating immune responses.

### *Origin of Dendritic Cells*

DCs form, together with monocytes and macrophages, a distinct leukocytic subgroup termed the mononuclear phagocyte system. Most subsets that are currently known originate from bone-marrow precursor cells, and differentiate depending on the selection of specific gene expression programs, as well as factors encountered in the cells' microenvironment.[25,26]

In the bone marrow, hematopoietic stem cells give rise to myeloid- (MPs) and lymphoid precursors (LPs). The MP lineage successively differentiates into macrophage/DC precursors which in turn split into monocytes and common DC precursors (CDPs), as well as some macrophage populations. The monocytic lineage encompasses two subsets, LY-6C$^+$ and LY-6C$^-$ cells, which enter the blood stream and populate multiple DC and macrophage subsets upon migration into peripheral tissues.[26] *In vitro* these monocytes are commonly used to generate monocyte-derived DCs, since they are easily obtained and come in relative high numbers. Differentiation towards DCs occurs in the presence of IL-4 and GM-CSF and is complete after 6 days.[27]

The CDP lineage on the other hand further differentiates into DC precursors (preDCs) or plasmacytoid DCs (PDCs) before leaving the bone marrow and entering the blood stream. Two subsets of blood circulating preDCs, also referred to as myeloid DCs, are currently recognized. Both populations show features of preDCs and immature DCs, lacking the characteristic 'dendrite phenotype', and effectively mature *in vitro* upon activation with TLR ligands or cytokine cocktails. Maturation of these DCs is accompanied by secreting cytokines and inducing T-cell proliferation.[26]

These blood circulating preDCs are therefore considered to be in transit, maturing into functional DCs only after entering the tissue. The CD1c$^+$ (BDCA-1$^+$) population appears to be the most abundant of the blood DCs, whereas CD141$^+$ (BDCA-3$^+$) DCs are known to be very rare. Functional studies have shown specific chemokine production by CD1c$^+$ cells, and identified CD141$^+$ cells as major producers of IFN-β. CD141$^+$ DCs were also shown to be capable of cross-presenting antigen for CD8$^+$ class 1 restricted CTL responses after TLR-3/CD283 ligation.[25,26]

Plasmacytoid (BDCA-2$^+$/BDCA-4$^+$) DCs and are the third blood DC population, and are also present in the bone-marrow and all peripheral tissues. Like the classical DCs, they are capable of antigen presentation and control of T-cell responses. What makes them unique is their relative long live span, and their specialization in the response to viral infections through massive productions of Type I Interferons (IFNs).[26,28]

*Maturation of DCs*

The DC life-cycle starts with bone marrow progenitors that give rise to DC precursors circulating in the blood. These precursors patrol the blood and lympatic system for invading pathogens. Differentiation into DCs is completed upon migration into peripheral tissues, where they reside in an immature state sampling the environment in search for microbes. Pathogen recognition and inflammation triggers the DCs to mature, up-regulating the expression of co-stimulatory molecules and inducing cytokine secretion. These mature DCs leave the peripheral tissues for migration to lymph nodes where they present the captured antigens to T- and B cells.[29]

Upon pathogen detection by PRRs, different intracellular signaling pathways are activated, leading to the transcriptional activation of a plethora of genes involved in DC maturation. These responses are generally mediated by the transcription factors nuclear factor-κB (NF-κB), activator protein 1 (AP-1), nuclear factor of activated T-cells (NFAT) and interferon regulatory factor (IRF). In inactive state, these factors can be found sequestered in the cytoplasm. Binding of PAMPs by PRRs induces a cascade of enzymatic reactions leading to the activation and nuclear translocation of these transcription factors, enabling transcription activation (figure 2).[30,31]

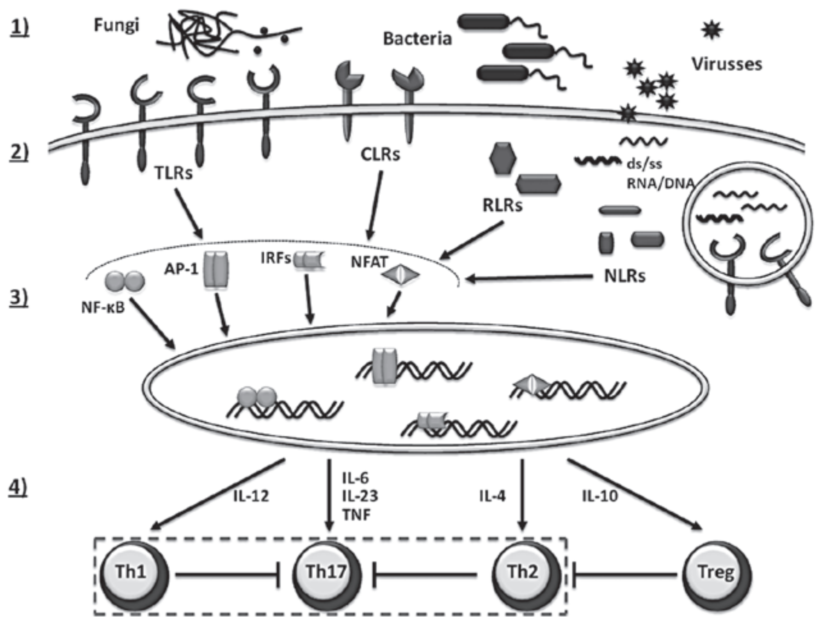


***Figure 2. Pathogen recognition and response by DCs.*** *Dendritic cells can detect many different pathogens through their cytoplasmic and membrane bound PRRs that recognize specific microbial conserved signatures. Upon binding, PRRs initiate intracellular signaling cascades that ultimately results in the activation of multiple transcription factors (TFs) in the nucleus. Here, these TFs regulate the expression of co-stimulatory molecule- and cytokine genes that are involved in T-cell activation. Depending on the pathogen and the PRR, different TFs can be activated, resulting in a differential cytokine expression and T-cell differentiation.*

These intracellular signaling events have been shown to differ depending on the PAMP and activated PRR, causing differential transcription factor activation. This results in expression of different target genes, thereby affecting the DC activation and maturation process. Regulation of cytokines IL-12 and IL-23 for instance, requires both common and distinct pathways. Activation of NF-κB and IRF5 is sufficient for the expression of IL-23, whereas IL-12 production additionally requires IRF1, -3 and -7 activation. IFN signaling, usually triggered by viral infections, stimulates IRF1, -3 and -7 activation, and therefore favors IL-12 production, which is important in anti-viral immunity. Conversely, detection of fungal pathogens via CLRs supports IL-23 secretion, thereby stimulating anti-fungal responses.[32,33]

Besides cytokine secretion, other molecules important in DC maturation are induced via this process. These include co-stimulatory receptors such as B7.1 (CD80), B7.2 (CD86) and CD40, as well as migratory molecules such as CCR7, that direct DC migration towards the lymph nodes. In contrast, the expression of adhesive receptors that retain the DCs in the peripheral tissues is abrogated via this process.[25]

*Regulation of immunity and tolerance*

Although macrophages and B-cells are also included in the term 'Professional Antigen Presentating Cells', or Professional APCs, DCs are by far the most efficient in presenting antigens to T- and B-lymphocytes. Not only are they at the start of immune responses against pathogens and tumors, they also protect the host against auto-immune diseases. This dual role is essentially achieved by the way DCs present antigens to T-cells, activating them to start an immune response, or to initiate tolerance. In essence, the activation state of the DC determines how T-cells are stimulated, and whether or not an immune response will be initiated.[29]

To date, four different subsets of Th-cells, generated through alternative co-stimulation, have been described, each with different specialized functions. Th1-cells function in responses targeting endogenous pathogens and tumor cells, whereas Th2- and Th17-cells stimulate immunity against extracellular microbes and viruses. Th2-responses are known to enhance the humoral immunity, and are involved in allergy development. Th17 proliferation is specifically induced during anti-fungal responses, and is negatively implicated in auto-immunity. The fourth subset are the Regulatory T-cells (Tregs), which regulate immune responses and prevent auto-immunity by maintaining peripheral tolerance.[34]

In DCs, presentation of extracellular derived antigens on MHC-II molecules to T-cells induces proliferation of $CD4^+$ Th-cells. Depending on the activated PRR and the type of pathogen, DCs secrete IL-12 for Th1-, IL-4 for Th2- or IL-23, IL-21 and IL-6 for Th17-responses (figure 2). Importantly, Th-cells activation also requires the expression of the co-stimulatory B7.1 and B7.2 molecules. DCs that express low to intermediate levels of B7.1 and B7.2, accompanied by IL-10 secretion, induce T-cell tolerance and stimulate the expansion of Regulatory T cell (Treg) (figure 1 and 2).[34,35]

Elimination of intracellular pathogens depends on activation of $CD8^+$ CTLs through MHC-I presentation of endogenous antigens. DCs are often not infected by intracellular pathogens, hence presentation of viral- or tumor derived antigens on MHC-I occurs via an alternative pathway, termed cross-presentation. This process is unique for DCs, and entails the phagocytic uptake of infected host cells, or tumor cells, followed by degradation and alternative routing of antigens to the cytoplasm, enabling presentation on MHC-I.[36] In addition, DCs also present the thus obtained antigens on MHC-II, accompanied by IL-12 secretion, activating anti-viral Th1-cell proliferation.

Hence, immune regulation greatly depends on DC maturation, which is directed by different factors present in the environment of the DCs. Naturally, the type of pathogens present is important to DC maturation, however, other factors such as inflammatory and immunosuppressive factors produced by other lymphocytes or tissues as well as hormones and vitamins also play a major role.

## NUCLEAR RECEPTORS

Cellular responses to hormones and vitamins are mediated by a specialized family of receptors termed Nuclear Receptors (NRs). The NR superfamily is a class of transcription factors that are crucial for many biological processes, including development, homeostasis and immune responses. In Men, 48 members have been identified and characterized. NRs are structurally conserved and consist of a variable N-terminal activation domain (activation function 1 or AF-1), a DNA-binding Domain (DBD) and a C-terminal Ligand Binding Domain (LBD). NRs are commonly divided into 3 subgroups, depending on their DNA- and ligand-binding characteristics.[37-39]

*Nuclear Receptor signaling*

The glucocorticoid- (GR), estrogen- (ER), progesterone- (PR), androgen- (AR) and the mineralcorticoid receptor (MR) are collectively referred to as steroid receptors, hormone receptors or Type I NRs, and are some of the first and most extensively studied NRs. Classically, these receptors reside in the cytoplasm complexed with chaperone proteins, and are activated upon hormone ligation (figure 3a). Ligand binding releases the NRs from the chaperone complex, allowing the receptors to homodimerize and translocate to the nucleus where they control expression of specific target genes. In addition, type I NRs have also been reported to function in the absence of ligand, and outside the nucleus.[40]

The Type II NRs, also indicated as vitamin receptors or retinoic x receptor (RXR) heterodimers, include the retinoic acid receptor (RAR), thyroid receptor (TR), vitamin

D3 receptor (VDR) and peroxisome proliferator-activated receptor (PPAR). In contrast to Type I NRs, Type II receptors are commonly present in the nucleus, bound to specific response elements in the promoters of their target genes. In the absence of ligand these RXR heterodimers bind corepressor complexes, preventing transcription. Upon ligand binding the corepressor complex is dissociated, and coactivator proteins are recruited, thereby allowing for gene transcription (figure 3a).[37-39,41]

The third group comprises NRs without identified cognate ligands, the orphan NRs. Well known examples are the estrogen receptor related receptor α (ERRα) and the retinoid acid-related receptor (ROR). Orphan receptors function in a ligand independent manner, however for some orphans, e.g. ERR ligand 1[42] and ERR,[43] natural ligands have been identified in subsequent studies.[37-39]

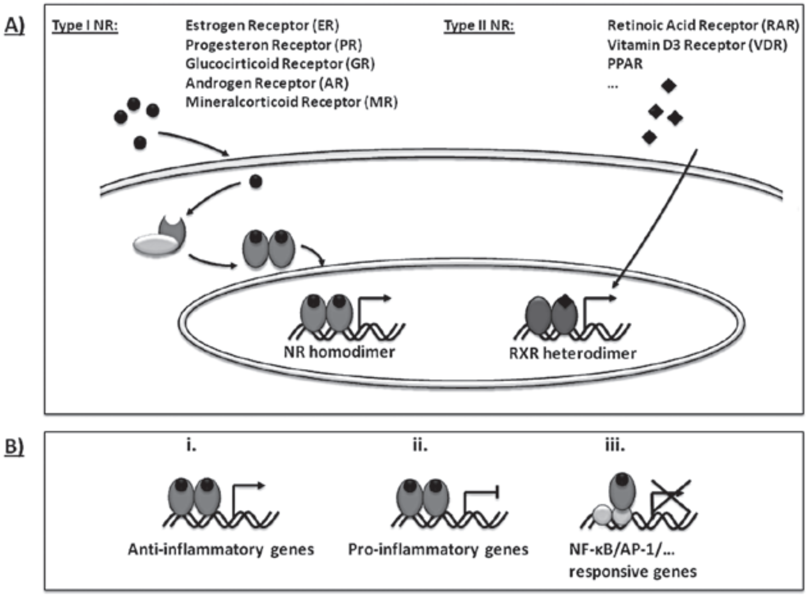


***Figure 3. Nuclear Receptor signaling.*** *(**A**) Type I NRs typically reside in the cytoplasm bound to chaperone proteins. Ligand binding triggers chaperone release, homodimerization and translocation to the nucleus where the NR activate specific target genes. Type II NRs on the other hand, are confined to the nucleus, complexed with NR co-repressors in the absence of ligand. Upon ligand binding the co-repressor complex is replaced by a co-activator complex, initiating transcription. (**B**) Three mechanisms by which the Type I NR GR exerts its anti-inflammatory effects. (i) GR directly induces expression of anti-inflammatory genes; (ii) GR directly inhibits expression of pro-inflammatory genes, and (iii) GR inhibits the activity of pro-inflammatory transcription factors.*

*Co-factors regulate Nuclear Receptor activity*

NRs play a role in diverse biological processes, turning different sets of genes on and off depending on the situation. However, whether gene transcription is induced or inhibited ultimately depends on the coregulator proteins recruited. NR coregulators are transcription regulators that do not bind DNA directly, but rather interact with NRs to mediate their transcriptional potency.[44] Since the cloning of the first NR coregulators, the field has expanded dramatically and currently encompasses more than 330 identified proteins (www.nursa.org).

Originally, co-regulators are categorized by their operational action: co-activators necessary for transcriptional activation, and co-repressors facilitating transcription repression. Later studies, however, have demonstrated repression by coactivators,[45] and vice versa,[46] suggesting that coregulator action depends on the gene, cell, and signaling context.[44] In general, DNA-bound NRs recruit coregulators depending on the presence of NR ligands. The most extensively studied NR corepressors are NCoR (Nuclear receptor CoRepressor) and SMRT (Silencing Mediator of Retinoic acid and Thyroid hormone receptors).[47,48] Recruitment to NR binding sites occurs in the absence of ligand, or in the presence of antagonists such as Tamoxifen (ER antagonist) or RU-486 (GR antagonist). In this corepressor complex, histon deacetylases (HDACs) prevent transcription by catalyzing chromatin condensation.[41]

The first authentic NR coactivator to be identified was SRC-1/ NCOA1,[49] which forms the SRC coactivator family together with SRC-2/TIF2/GRIP1/NCOA2 and SRC-3/AIB1/ACTR/pCIP/RAC3/NCOA3. Agonist binding by NRs leads to attraction of coactivators and the assembly of a coactivator complex. HDACs are exchanged for histon acetyltransferases (HATs), which facilitate local acetylation, creating a transcriptionally permissive environment at the promoter.[41]

*Nuclear Receptors and Dendritic Cells*

Nuclear Receptors are increasingly recognized as immune-regulatory receptors, having positive and negative regulatory activities in immune cells. Anti-inflammatory functions of GR, VDR, and LXR, have been documented, whereas ER, PPARγ and RARα were shown to have both stimulatory and inhibitory effects on immune cells.[50-53] NR signaling plays a major role in DCs, which express 20 out of 48 known NRs, affecting both differentiation and maturation. In fact, DCs have been demonstrated to actively convert hormone precursors into active NR ligands.[54]

During DC differentiation from human monocytes, expression of both PPARγ and LXRα has been shown to be upregulated.[55] Stimulation with the PPARγ ligand rosiglitazone during DC differentiation results in DCs with enhanced phagocytic activity, and reduced IL-12 secretion upon maturation with LPS, promoting Th2 over Th1-responses. In addition, these cells have enhanced lipid antigen presentation capacities, important for activation of invariant natural killer T-cells.[56] Activation of LXR, a type II NR that binds oxidized forms of cholesterol,[57] has been shown to reduce the capacity of DCs to activate T-cells.[58] Furthermore, exposure to estrogens or derivatives of vitamin A (retinoids), stimulates the differentiation and activation of monocytes to DCs via IRF4[59] or activation of RAR and RXR receptors,[60] respectively.

DC maturation can also be modulated by NR stimulation. The majority of the currently known consequences of NR activation encompasses impaired DC maturation and T-cell stimulation. In some cases, however, stimulation of DCs with NR ligands has been shown to enhance DC mediated immune activation. The best known example are retinoids, which enhance T-cell stimulation by LCs, promote DC maturation by enhancing DNA binding of NF-κB, and stimulate IL6 and TGFβ expression in moDCs, inducing a mucosal DC phenotype. In contrast, RA can also induce apoptosis in developing DCs, via RARα-RXR activation.[61] Studies where DCs were exposed to estrogens produced mixed results, demonstrating both immunostimulatory and -suppressive effects.[50,62,63]

More extensively studied are the suppressive effects of NR signaling on DC function. Exposure to GR, VDR or LXR ligands potently suppress the capacity of DCs to induce inflammation, and instead trigger tolerance.[58,64,65]Corticosteroids, the ligands for GR, are particularly appreciated for their tolerizing effects, and are widely used as immunosuppressive drugs. Three important mechanisms by which GR exerts its repressive effects are currently recognized (figure 3b).[66-70] *First*, ligand-bound GR homodimers directly induce transcription of immune suppressive genes by binding to their promoters. In DCs, GR activation induces the expression of the glucocorticoid induced leucine zipper (GILZ), which potently suppresses multiple intracellular signaling pathways that aid immune activation.[71] Other important GR targets with immune suppressive functions include IL-10, lipocortin-1, and DUSP1 (MKP1).[66,72] *Second*, GR homodimers can potentially inhibit expression of immune stimulatory genes via negative GREs (nGREs). The nGRE consensus sequence has recently been identified by Surjit *et al.* (2011), however, the exact role in mediating GR dependent immune suppressive effects remains unclear.[73] In the *third* mode of action GR acts in monomeric fashion, binding to DNA-bound transcription factors that induce the expression of immune stimulatory genes, and repressing their transcriptional activity. Well known examples include the NF-κB and AP-1 complexes, as well as IRF3 and CREB transcription factors. Together, these responses efficiently inhibit expression of pro-inflammatory genes, creating tolerogenic DCs (tolDCs). These tolDCs have impaired MHC class I and II presentation, suppressed costimulatory properties and therefore repress T-cell responses and induce Treg proliferation.[74-78]

Importantly, DCs can also convert hormone precursors into their active form, a process which otherwise occurs in the liver and the kidneys. Secretion of active NR ligands by DCs is important for local immune regulation, without affecting immune responses in the rest of the body. In the gut, DCs convert vitamin A into RA, which is important in the prevention of responses against beneficial microbes. RA, in combination with other compounds, blocks IL-6 and TGF-β secretion by Th17-cells and stimulates Treg differentiation, inducing gut tolerance. In addition, DC produced RA instructs T-cells to migrate to the gut via the upregulation of gut-homing receptors. Similarly, DCs in the skin release the active form of vitamin D, instructing local T-cells to induce CCR10 expression and migrate to the epidermis.[54]

*NRs in tumor biology*

Apart from regulating immune responses, NRs have also been implicated in carcinogenesis. Several Type I and Type II NRs have crucial roles in tumor biology, and are important targets for drug-based therapies. In particular breast- and prostate carcinomas are known for their hormone sensitivity, however, other tumors have also been found to be subjected to NR signaling. In fact, NRs are very likely to be involved in all known carcinomas, considering their important role in cell cycle and differentiation.[51,79]

In breast carcinoma cells, the Type I NRs ER and PR are known to stimulate cell proliferation and tumor growth, whereas the Type II NRs RAR-RXR and PPAR-RXR induce differentiation and inhibit proliferation through cell-cycle arrest and apoptosis. Breast cancer patients are therefore checked for NRs expression status, and treated accordingly. Although RA based therapies have limited efficacy due to induced RA resistance in tumor cells, treatment with ER blocking agents has proved to be highly effective.[80,81]

Prostate cancer is also known as a high incidence, hormone responsive cancer, and is dependent on activation of the Type I NR AR. Treatment generally aims to suppress AR activity via androgen deprivation and administration of AR antagonists.[82] In addition, the Type II NR VDR is implicated in growth inhibition of prostate tumors, exerting anti-proliferative effects and inducing differentiation in prostate cancer cells.[83]

Besides breast- and prostate carcinoma, many more hormone responsive tumors have been described, including different kinds of leukemia, ovarian cancer, pancreatic cancer and colon cancer.[84-87] Importantly, alongside NRs, also their co-regulators have been implicated in cancer development, and their expression levels are often used to predict treatment efficacy and survival.[88]

## DC-SCRIPT: DC ASSOCIATED MOLECULE AND NR COREGULATOR

A link between regulation of NR function, DC and tumor biology can be found in the transcription regulator DC-SCRIPT (DC-Specific transCRIPT), also known as ZNF366. Within the immune system, DC-SCRIPT has been described to be specifically expressed by DCs. Expression of DC-SCRIPT mRNA was detected in cultured moDCs as well as freshly isolated MDCs, PDCs and LCs. Other cells of the immune system, including T-cells, B-cells and macrophages, were tested negative for DC-SCRIPT mRNA.[89] Outside the immune system, DC-SCRIPT expression was demonstrated in healthy and malignant epithelial cells from the breast and prostate, albeit expression was severely reduced in carcinoma cells. Within these cells, DC-SCRIPT was demonstrated to interact with NRs, and affect their transcriptional activity. Together these findings suggest an important role for DC-SCRIPT in DC and tumor biology, via the regulation of NRs.[90,91]

*Structure and function*

The *DC-SCRIPT* gene is positioned on chromosome 5q13.2,[92] and encodes an 8 kb long mRNA. Five exons are recognized within the mRNA sequence, encoding a single Open Reading Frame (ORF) of 2232 nucleotides in length. Protein translation starts from the ATG codon at position 448 in exon 2, and continues untill the TGA translational stop codon in exon 5. The resulting protein contains 744 amino acids (aa) encoding a proline-rich domain (aa 111-219), 11 $C_2H_2$ zinc finger motifs (aa 255-556) and an acidic region (aa 586-690). In addition, a N-terminal Nuclear Localization Signal (NLS), as well as acidic-region located CtBP- and NR interaction motifs have been recognized within the DC-SCRIPT protein. Post-translational modifications (PTMs) have been suggested for DC-SCRIPT, based on the presence of possible phosphorylation-, SUMOylation and *N*-glycosylation sites.[89]

DC-SCRIPT expression was demonstrated in human and mouse, and predicted in many other species including rat, chimpanzee, chicken and pufferfish. Sequence homologies demonstrate that *DC-SCRIPT* is a well conserved gene, with the protein sequence being >80% identical between men and mice. In particular the zinc-finger domain shows high levels of resemblance between human- and mouse-, and human- and pufferfish proteins, showing respectively 93% and 98,5% homology.[89,93]

The presumed transcription factor function of DC-SCRIPT is deduced from the presence of its 11 zinc-fingers. These structures are known to mediate protein-DNA, protein-RNA or protein-protein interactions, and are found in many transcription factors. The classical Cys-Cys:His-His zinc fingers that are present in DC-SCRIPT, mostly aid protein-DNA interactions, suggesting a direct role in regulating target gene expression. The conserved character of the zinc finger region suggests that DC-SCRIPT binds similar DNA sequences in different species, thereby regulating the same genes.[89,93]

Furthermore, a direct interaction between the transcriptional corepressor CtBP1 (C-terminal Binding Protein 1) has been demonstrated, concomitant with the presence of CtBP-binding sites within the acidic domain of DC-SCRIPT. CtBP1 acts as corepressor through the recruitment of HDACs to the site of transcription, thereby catalyzing chromatin condensation. Hence DC-SCRIPT can have a repressive effect on gene transcription, through interaction with CtBP1.[89,93] Finally, DC-SCRIPT suggestively functions as NR coregulator, as it contains the NR interaction LXXLL motif, which is regularly found in NR co-activators.[94]

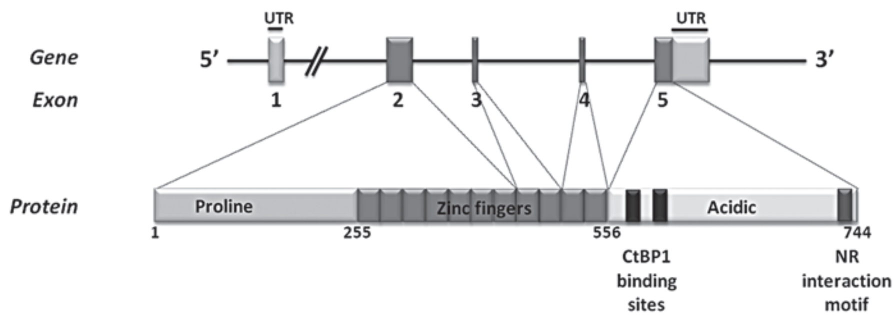


***Figure 4. DC-SCRIPT gene and protein.*** *The DC-SCRIPT gene contains 5 exons which encode for; the 5'-UTR (1); the proline domain and zinc-fingers 1-7 (2); zinc-fingers 8-11 (3 and 4); the acidic domain (5). The DC-SCRIPT protein contains 744 amino acids, and contains two CtBP1-, and a LxxLL NR interaction motifs.*

### *Function in DCs and coregulator of NRs*

Expression of DC-SCRIPT has been demonstrated in all DC subsets tested to date, in contrast to other DC associated molecules. In moDCs, DC-SCRIPT mRNA was readily detected during differentiation of monocytes to DCs with IL-4 and GM-CSF, and found to be consistently expressed throughout moDC life. Stimulation of monocytes with GM-CSF alone on the other hand, did not result in DC-SCRIPT mRNA expression.[89]

Interestingly, DC-SCRIPT expression is potentially regulated by different transcription factors that have a profound role in hematopoiesis and DC biology. These factors include growth factor independence (Gfi), GATA-1, AP-1, Spi-B, NF-κB and c-Rel, and their binding sites can be found clustered in the 300 bp preceding the first exon of DC-SCRIPT.[93]

DC-SCRIPT protein expression in DCs was found to be confined to the nucleus, in agreement with its presumed role as transcription factor. Furthermore, DC-SCRIPT was suggested to functionally interact with CtBP1 in DCs, as both proteins were found to colocalize within the nucleus of moDCs. Hence repression of DC-SCRIPT target genes in DCs is possibly mediated through interaction with CtBP1.[89]

Recently, DC-SCRIPT expression was demonstrated in breast- and prostate epithelial cells, where it importantly functions as NR co-regulator. Interaction with multiple NRs, including Type 1 ERα, PR, AR and GR and Type II RARα, VDR and PPARγ has been demonstrated, resulting in either repression or stimulation of the NR transcriptional activity. Hereby, DC-SCRIPT balances NR activity, preventing carcinogenesis.[90,91]

## SCOPE OF THIS THESIS

In this thesis we have further elucidated the role of DC-SCRIPT in both human dendritic cells and breast carcinoma cells. DCs are at the centre of the immune system and play an essential role in the initiation and regulation of immune responses. Advanced molecular understanding of the processes in these cells can provide important insights into their nature and functionality, knowledge that can potentially be used in the development of effective immunotherapy with DC-vaccinations.

DCs can discriminate between many different types of pathogens via their PRRs. Crosstalk between these PRRs is essential for tailoring responses to provide the most effective immune response. This process is explained in more detail in **chapter 2**, which describes the crosstalk between PRRs that are involved in the detection, and response to fungal pathogens.

In **chapter 3** we studied the role of DC-SCRIPT in the regulation of TLR induced cytokine production during dendritic cell maturation. DC-SCRIPT expression was investigated in different DC subsets, and knock-down of DC-SCRIPT expression resulted in an increased IL-10-, and a consequent decreased IL-12 secretion upon TLR-4 or -7/8 mediated maturation.

**Chapter 4** further elucidates the function of DC-SCRIPT in DCs by focusing on another important factor in DC maturation, the glucocorticoid receptor. Activation of GR prior to DC maturation leads to the generation of tolerogenic DCs, which is importantly mediated by the GR target gene GILZ. In this chapter, we show that DC-SCRIPT functions as co-repressor on GR mediated transcription. Moreover, we found that DC-SCRIPT silencing resulted in enhanced GILZ expression, strengthening the suggesting for an important role for DC-SCRIPT in DC maturation.

A function for DC-SCRIPT as nuclear receptor co-regulator was also investigated in breast carcinoma cells, described in **chapter 5**. In these cells, DC-SCRIPT was found to co-repress the proliferation stimulating NRs ER and PR, while exerting a co-activator function on the anti-proliferative NRs RAR-RXR and PPAR. Concomitant with these findings, DC-SCRIPT expression was found to be a prognostic marker for disease-free survival.

NRs play an important role in both dendritic cell- and breast cancer biology, and cross-talk between these receptors is an important aspect of their function. **Chapter 6** reviews the current knowledge on NRs crosstalk in breast carcinoma cells, and anticipates on a role for DC-SCRIPT in this process, regulating both Type I and Type II NRs.

**Chapter 7** summarizes the findings of this thesis, and discusses their implications and future perspectives in DC-, NR-, and cancer biology.

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# Chapter 2

## Molecular view on PRR crosstalk in antifungal immunity

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## ABSTRACT

The identification of a major class of innate immune receptors, termed Pattern Recognition Receptors (PRRs), has boosted research on innate pathogen recognition. The immune response to a specific pathogen is not restricted to the recognition by one type of PRR or activation of a single cell type, but instead comprises complex collaborations between different receptors, cells, and signal mediators. Here we will discuss the crosstalk between PRRs involved in fungal recognition, focusing on the molecular interactions occurring at the plasma membrane.

## INTRODUCTION

In multicellular organisms, the innate immune system is the first line of defence against invading pathogens. Recognition and uptake of these microbes is crucial for an effective host defence, and is facilitated by phagocytes such as neutrophils, macrophages and dendritic cells (DC). Phagocytosis of microorganisms by neutrophils triggers direct cellular antimicrobial immunity such as production of reactive oxygen species (ROS) and the fusion of cytoplasmic granules with pathogen containing vacuoles.[1] This mechanism is highly effective in killing most bacteria and fungi. Furthermore, recognition of pathogens by phagocytes activates intracellular signalling pathways that result in production of numerous cytokines and chemokines. These mediators attract more phagocytes and activate antigen presenting cells (APC) such as DC, creating an essential foundation for the initiation of adaptive immunity, that protects us from re-infection.

Despite its importance, in the past the innate immune system has received relatively little attention. The identification of the innate immune receptors termed Pattern Recognition Receptors (PRRs) provided a boost for research on innate pathogen recognition. Indeed, proper pathogen recognition is key to adequate immune defence, and is facilitated by PRRs expressed on phagocytes. By definition, these germline-encoded receptors detect Pathogen Associated Molecular Patterns (PAMPs), structures conserved among microbial species. Well known PAMPs include the bacterial components lipopolysaccharide, peptidoglycans and flagelin, viral nucleic acids and fungal polysaccharides such as mannan and β-glucans. Currently, PRRs are categorised into four classes: i) Toll-Like Receptors (TLRs), ii) C-type lectin receptors (CLRs), iii) Retinoic acid-inducible gene (RIG)-I-like receptors (RLRs) known also as RIG-I helicases (RLHs) and iv) the NOD-like receptors (NLRs) (see: BOX 1).

In the past, the majority of *in vitro* studies aiming to unravel the mechanism of pathogen recognition by PRRs used an isolated cell type or a single PAMP. In recent years it has become increasingly apparent that the immune response to a specific pathogen is not restricted to the recognition by one type of PRR or activation of a single cell type, but instead comprises complex collaborations between different receptors, cells, and signal mediators. Indeed, *in vivo* invading pathogens generally contain multiple PAMPs that are recognized by various PRRs on multiple cell types. Additionally, there is redundancy in the recognition of PAMPs between different classes of PRRs. The simultaneous or sequential binding of multiple PRRs to different PAMPs resulting in coordinated activation or inhibition of signal transduction is referred to as 'receptor crosstalk'. It is anticipated that the presence of PRRs in multi-receptor complexes in the plasma membrane facilitates this crosstalk.

**BOX 1. Four classes of pattern recognition receptors**

**TLRs** are the best characterized PRRs. These type I transmembrane proteins are present on the plasma membrane or within the endosomal compartment. The extracellular domain is involved in the recognition of PAMPs, while the intracellular TIR domain activates signalling pathways leading to the activation of NF-κB. So far, 10 functional TLRs have been identified in man versus 12 in mice. Each TLR detects different PAMPs from viruses, bacteria, fungi, and parasites.[2] Some TLR, such as TLR2 form functional heterodimers, thus further broadening PAMP recognition.
**CLRs** represent the second class of PRRs, mainly recognizing carbohydrate ligands. The CLR family comprises transmembrane- and soluble receptors that share a carbohydrate-recognition domain (CRD).[3] Not all CLRs effectively function as PRRs. Those which do, can be divided into two classes: "self-sufficient" CLRs that autonomously recognize PAMPs initiating downstream signalling, and CLRs that do recognize PAMPs but are dependent on partnering proteins for intracellular signalling.
**RLRs** are present in the cytosol and involved in sensing intracellular pathogens. RLRs bind mainly viral nucleic acids. So far, three members of the RLR family have been identified: RIG-I, MDA-5 and LGP2. RIG-I and MDA-5 recognize a wide range of viruses and initiate IFN responses, whereas LGP2 is considered to regulate responses initiated by RIG-I and MDA-5.[4]
**NLRs** are also expressed intracellularly. Members of the NLR family have a common domain architecture that includes a nucleotide-binding domain (NBD) and a leucine-rich repeat (LRR) domain. The best characterized NLRs are NOD1 and NOD2. They sense different building blocks of peptidoglycans (PGN). Some NLRs can function as crucial components in multiprotein complexes termed "inflammasomes" that are important for activation of inflammatory caspases and cytokines of the IL-1 family.[5]

As a consequence, separate or shared signalling pathways are activated that ultimately determine the type and magnitude of the immune responses directed against the pathogen.

The outcome of PRR crosstalk may be quantitative when receptors act synergistically or antagonistically. Synergistic responses may be particularly important under conditions of low ligand concentrations, when recognition by more than one receptor can enhance the immune response. A classical example of the synergistic response is the production of IL-12p70 cytokine by DCs, which increases greatly when multiple PRRs are triggered.[6] On the other hand, collaboration of PRRs

is also important to dampen unwarranted host responses. For example triggering of the C-type lectin receptor, DCIR, selectively inhibits TLR8-meditated IL-12 and TNF-α production.[7]
Crosstalk between distinct PRRs can also have a qualitative effect. For instance, recognition of *Salmonella typhimurium* by TLR4 and TLR5 activates transcription of proinflammatory cytokines including IL-1β. Subsequent release of functional IL-1β from the cells, however, requires processing of pro-IL-1β by Caspase-1, which is critically dependent on activation of NLRP3 and NLRC4 in the inflammasome.[8]

Hence, collaboration between PRRs enhances the specificity of recognition, broadens their signalling capacity and enables the host to detect and respond to almost any type of infection. In this review we will focus on the molecular basis of PRR crosstalk important in detection of the fungal pathogen *Candida albicans*.

## FUNGAL RECOGNITION BY THE INNATE IMMUNE SYSTEM

The last decade has yielded significant advances in the identification and functional characterization of a variety of PRRs that sense fungi. *Candida albicans* represents an opportunistic fungus that asymptomatically colonizes the mucosa of most healthy individuals. However, mucosal and/or systemic infections caused by *C. albicans* are regularly observed in immunocompromised patients, emphasizing the importance of the immune system in clearing fungal pathogens. The severity of such infections is exemplified by the high mortality rate amongst patients with invasive candidiasis, despite the availability of novel effective antifungal drug classes such as the azoles and echinocandins.

The cell wall of *C. albicans* is comprised of different sugar polymers (chitin, β-glucans, mannan) and proteins that can function as PAMPs for CLRs, TLRs, and NLRs.[9,10] In addition, members of the scavenger receptor (SR) family and certain integrins also possess intrinsic fungal recognition capacity although these are not classified as classical PRRs. Many PRRs that are involved in *C. albicans* recognition belong to the CLR family, (reviewed in [11]) and can directly activate signalling pathways, or indirectly through interaction with other signalling adaptors or receptors, such as the FcRγ chain or TLRs.[12] The importance of CLR in anti-fungal immune responses has been validated in different murine knock-out models (reviewed in [10,11]).

The recent finding that human Dectin-1 deficiency causes an increased susceptibility to mucocutaneous fungal infections emphasizes the importance of specific CLR *in vivo.*[13] Furthermore, individuals with mutations in the CLR adaptor molecule CARD9 are more susceptible to both mucosal and systemic fungal infections.[14] Dectin-1 is expressed by DCs, macrophages and monocytes, where ligation with its ligand β-1,3 glucan induces phagocytosis and an oxidative burst, as well as the production of eicosanoids, inflammatory cytokines and chemokines. Albeit β-1,3 glucans in de cell wall of *C. albicans* are only exposed in bud scars or after heat inactivation of the yeast, Dectin-1-knockout mice were documented to have increased susceptibility for *C. albicans* infections.[15] However, it must be noted that different results were obtained between the mice models Balb/cA and 129/C57BL/6, most likely due to background differences in inherent T-cell polarisation.[16,17] In addition, differences can arise between systemic versus mucosal *C. albicans* infections. Dectin-1 is mainly involved in mucosal candidiasis, which is evidenced by the study of Ferwerda et al (2010). Interestingly, Dectin-1 molecules in myeloid cells have recently been reported to cluster in a 'phagocytic synapse' crucial for triggering phagocytosis and full antifungal activity.[18] Responses following Dectin-1 ligation are variable depending on cell type and microenvironment, however, prevailing evidence suggest that Dectin-1 signalling preferential directs Th17 polarization.[19] Similar Th17-polarizing effects have been described for other CLRs such as dectin-2 and MR.[20,21]

Fungal recognition by the TLR family can be mediated by TLR2/6, TLR4, and TLR9 sensing phospholipomannan (PLM), O-linked mannan, and phagocytosed fungal DNA, respectively.[10] The latter, however, seem to have a redundant role, since TLR9-deficient mice do not show increased susceptibility to *C. albicans* infection.[22] The role of the individual receptors has not been fully elucidated and divergent results have been published, however, it is generally accepted that TLR-mediated antifungal immunity acts through the induction of inflammatory cytokines. Deletion of the intracellular TLR-adaptor protein MyD88 clearly increases susceptibility to fungal infections (reviewed in [10]). Moreover, *TLR4* polymorphisms in humans have been found to associate with an increased risk of invasive fungal infections.[23,24] In contrast, TLR2-deficient mice are more resistant to disseminated candidiasis, showing enhanced Th1 responses and decreased Treg proliferation.[25,26] A similar, although weaker effect, was shown in TLR6-deficient mice, suggesting both TLR2 and TLR6 are involved in *C. albicans* detection.[27]

Recent studies have demonstrated an important role for the NLRP3 inflammasome in anti-fungal immunity.[28] *C. albicans* can induce caspase-1-mediated IL-1β secretion in a NLRP3 dependent-manner in APC, most likely through Dectin-1/Syk kinase/CARD9 signalling.[29,30] Interestingly, a critical role for the inflammasome in regulating Th17/Th1 responses during *C. albicans* infection was recently reported.[31] Other receptors involved in *C. albicans* detection include the family of scavenger receptors (SR), Integrins and Fc Receptors (FcR). SR represent structurally unrelated proteins that recognize multiple ligands including lipoproteins, β-glucan motifs, microbial antigens, and modified or endogenous molecules derived from the host.[32] Integrins on the other hand can act as opsonic receptors that recognize fungal particles coated with complement factors, or possess intrinsic microbial recognition. Fc receptors (FcR) recognize antibody-opsonised *C. albicans* and can also efficiently induce phagocytosis, cytotoxicity and/or antigen presentation.[33,34]

Recognition of *C. albicans* by these many different PRRs expressed by innate immune cells is a highly complex and dynamic process. Evidence is accumulating that the clustering of receptors, including PRR, into organized membrane complexes, such as lipid rafts or tetraspanin microdomains, is important to regulate ligand binding and subsequent signal transduction.[35-37] This may allow for cross-talk between the different PRRs that facilitates integration of different incoming signals leading to a potent antifungal response.

## MOLECULAR VIEW ON PRR CROSSTALK IN FUNGAL RECOGNITION

Collaboration between different classes of PRRs is important for the innate immune responses to *C. albicans*. The underlying molecular mechanisms, however, are still largely unknown. Interaction studies have been performed for some of these PRRs, and suggest physical contact between some of the receptors at the plasma membrane. True insight into the molecular interaction platforms involved in PRR collaboration requires more detailed characterization of these interactions, and should distinguish between direct and indirect binding. Here we will review the current understanding of the molecular interactions among PRRs at the plasma membrane that are involved in fungal recognition.

### *TLR-2/TLR-6*

TLR2 is known to form heterodimers with TLR6 in the recognition of fungal PAMPs. This interaction is well characterized at the molecular level with known crystal structure and binding domains. Dimerization of TLR molecules occurs at both the extracellular Leucin-rich repeat domain (LRR) and the intracellular TIR-domain. The LRR domain forms the characteristic TLR horseshoe structure, consisting of the 24 amino acids conserved motif XLXXLXXLXLXXNXLXXLPXXXFX. Interestingly, TLR2 has an aberrant LRR domain that lacks the conserved Asparagine ladder important for structure stability. It has been suggested that this modification allows for variations in structural conformation, permitting the binding of different ligands and receptors. Intracellularly, TIR-TIR interactions depend on the BB-loop, DD-loop and αC-helix, domains that are essential for recruitment of adaptor proteins and domain stability. Indeed, mutations within the BB-loop region have been shown to abrogate TLR signalling. For TLR2/6 complexes, the adaptor proteins MyD88 and Mal directly interact with the TIR domains, mediating downstream signalling leading to NF-κB activation and cytokine production.[38] In addition, TLR2 can also functionally cooperate with the CD14 receptor, whereby CD14 enhances TLR2-mediated NF-κB activation in response to zymosan.[39] Whether a direct interaction of the TLR2 heterodimers with CD14 is essential for this collaboration is unknown.

*Dectin-1*

A molecule well known to functionally synergize with TLR2 is Dectin-1. This CLR family member is composed of an extracellular carbohydrate recognition domain (CRD), a short stalk region, a transmembrane domain and a 40 amino acid long intracellular tail. Alternative splicing generates respectively two and eight isoforms in mice and men, with both species preferentially expressing the 'stalkless' Dectin-1 isoform. The extracellular C-type lectin domain is used for β-glucan binding. Ligation of β-glucans triggers phosphorylation of the Dectin-1 intracellular tail, containing an unconventional immunoreceptor tyrosine-based activation motif (ITAM). Conventional ITAM motifs contain two tyrosine phosphorylation sites that are phosphorylated upon receptor activation. Syk family kinases bind to these phosphorylated sites via two Src homology 2 (SH2) domains, forming the basis of the intracellular signalling route. In the Dectin-1 ITAM-like motif, the membrane-distal phosphorylation site is not available for binding to an SH2 domain due to an additional amino acid. However, Syk recruitment and activation requires both SH2 domains to bind phosphorylated tyrosine residues. For Dectin-1 this is only possible by receptor dimerization, providing a binding site for Syk on adjacent clustered Dectin-1 ITAM motifs. Syk subsequently recruits the CARD9/Bcl10/Malt1 complex, leading to cytokine secretion via the activation of Erk, p38 and Jnk MAP kinases, and NF-κB and NFAT transcription factors.[19] Dectin-1 is known to form homodimers and larger clusters in the plasma membrane in order to induce signalling, and has been shown to synergistically collaborate with different TLRs, including TLR2. Despite many attempts, no direct interactions between Dectin-1 and TLR2 have been reported to date.

*Galectin-3*

A molecule possibly linking Dectin-1 and TLR2 molecules is Galectin-3. This S-type lectin receptor belongs to the Galectin family, defined by their conserved elements in the CRD domain. In mammals, 15 members of the Galectin family have been identified, all of which are synthesized and stored in the cytoplasm, separated from their glycan ligands. Upon infection these molecules are released, and function as soluble PRRs or immunomodulators. Galectin-3 contains one CRD and an additional non-CRD domain, which functions in oligomerization. The unbound soluble Galectin-3 is found in monovalent form. Ligand binding induces oligomerization through self-assembly of the N-terminal non-CRD domain, generating pentameric Galectin-3 molecules with multivalent CRDs. Galectins lack a transmembrane domain or signalling motif, but are implicated in direct binding of host glycoproteins, crosslinking receptors and ligands at the cell surface. Indeed, the formation of so-called Galectin-3 lattices has been shown to promote cell-surface retention of cytokine and growth factor receptors, by interfering with endocytosis. This potentially results in prolonged signalling and facilitates receptor collaboration. Importantly, since Galectin-3 binds β-1,2 oligomannans of *C. albicans*, it can also directly crosslink pathogens to this receptor complex.[40]

Interestingly, Esteban *et. al* (2011) recently discovered a physical association between Dectin-1 and Galactin-3 in the cell membrane of murine macrophages using co-immunoprecipitation studies. Stimulation with zymosan particles increased the amount of Dectin-1 in the immunoprecipitate.[41] In line with this work, crosstalk between TLR2 and Galectin-3 has been reported, which enhanced TLR2-induced TNFα production. Again, co-immunoprecipitation studies demonstrated an interaction between endogenously expressed TLR2 and Galectin-3 in THP-1 cells, which was critically dependent on prior stimulation with *C. albicans.*[42] Unfortunately, secretion of other relevant cytokines such as IL-6, IL-23, IL-10 and IL-12 were not assessed, despite clear effects on TNFα. This could provide a more complete view on the functional role of Dectin-1-Galectin-3 and TLR/2-Galectin-3 collaboration in *C. albicans* detection.

Further insight into the molecular make-up of putative Dectin-1, Galactin-3 and TLR2 complexes requires the identification of the domains involved in the molecular interactions. Both studies use stringent co-immunoprecipitation conditions, suggesting direct interactions. Taken together, these results suggest that Galectin-3 represents an important mediator in the recognition of *C. albicans* by molecular assembly of TLR2 and Dectin-1 in the plasma membrane at the site of fungal recognition. An important question that remains to be answered is how clustering of these receptors during *C. albicans* detection occurs in time. Possibly, initial binding could be facilitated by coating of fungal material with Galectin-3. This could facilitate ligand binding by Dectin-1 and TLR2/6, as well as recruitment of other cell surface receptors, which will induce intracellular signalling and will stabilize the interaction. Finally, phagocytosis and intracellular degradation may release ligands for intracellular PRRs such as TLR9. Hence, an interesting challenge lies in assessment of the sequential events leading to receptor activation and clustering during *C. albicans* detection.

*Tetraspanins*

Another phenomenon contributing to crosstalk between different PRRs in fungal detection is the occurrence of specialized membrane microdomains controlling signal transduction and cell function. The tetraspanin family of transmembrane-four proteins have the ability to interact *in cis* with specific (immune-) receptors, with each other, and with signalling molecules, whereby they form multi-molecular complexes, or 'tetraspanin microdomains'.[43,44] There is now convincing evidence that tetraspanins in immune cells control proliferation, antibody production and antigen presentation (reviewed in [45,46]). Moreover, tetraspanins are involved in the pathogenesis of infectious diseases.[47,48] Two independent studies have shown that the tetraspanins CD37 and CD63 interact with Dectin-1 in the cell membrane of human and murine APC.[49,50] Although the exact binding domains have not been identified, the stalk region present in the larger isoform A of Dectin-1 was not required for the interaction with CD37 since co-immunoprecipitations were successfully performed with isoform B of Dectin-1. CD37 was demonstrated to stabilize Dectin-1 expression at the plasma membrane of macrophages and to inhibit Dectin-mediated signalling leading to IL-6 production. This effect was specific for Dectin-1, because signalling via other PRR (including TLRs) was not affected by CD37-deficiency.[50] Thus, tetraspanins can modulate the organization and subsequent downstream signalling of specific PRR by their recruitment into tetraspanin microdomains leading to immune activation or tolerance.[36] It will be intriguing to investigate how the Dectin-1-tetraspanin interaction relates to the reported collaboration between Dectin-1 and TLR2, and moreover whether tetraspanins modulate Syk- or Raf-1-dependent signalling pathways which are known to be active downstream of Dectin-1

*Crosstalk between other PRRs involved in C. albicans detection*

Different studies have demonstrated that also other CLR, including the mannose receptor (MR), DC-SIGN (SIGNR1 in mouse) and Dectin-2 can collaborate with other fungal sensors during antifungal responses. For example, Dectin-2 was reported to interact with the FcγR chain, which was essential for the detection of *C. albicans* hyphae and the subsequent induction of TNFα and IL-1 receptor antagonist (IL-1Ra).[51] The intracellular domain of Dectin-2 (amino acids 8–14) proximal to the transmembrane domain was required for the association with the FcγR chain. Other studies report functional interactions between Dectin-1 and DC-SIGN/SIGNR1 during *C. albicans* recognition although the underlying molecular mechanisms are ill-defined.[52,53] Similarly, TLR4 collaborates with Dectin-1 in APC as has been shown for TLR2.[54] Finally, CR3 and also the recently discovered CLR Mincle interacts with Fc receptors in the plasma membrane of APC during *C. albicans* detection.[55]

Together, clustering of PRRs importantly aids to the formation of the 'phagocytic synapse', providing a platform for efficient phagocytosis of the captured antigen.[18,56] However, the amount and type of PRR ligands that are available to immune cells can differ between initial interactions, full blown infections and the type of fungus. Initially, only surface expressed PAMPs on the intact pathogens trigger PRR detection. During ongoing infections, however, more ligands become available through the degradation of fungal material. The quality and quantity of PRRs triggered on immune cells may therefore differ during the development of a fungal infection, possibly resulting in tuning of responses.

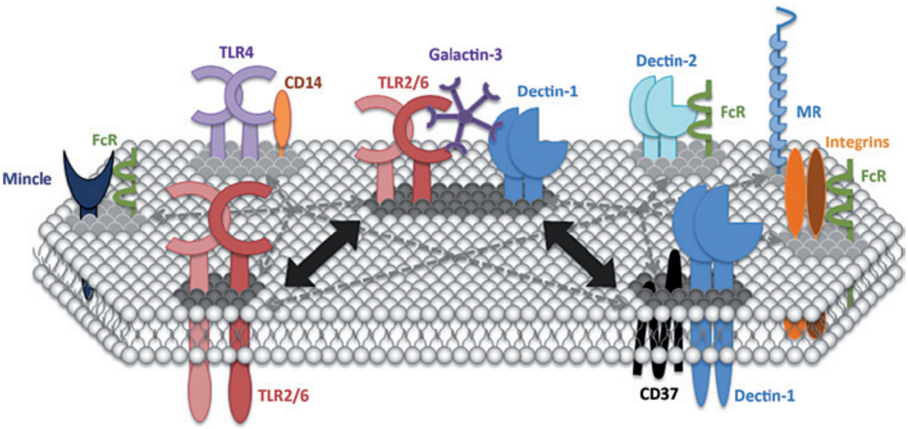


***Figure 1. Model of the molecular interactions and collaborations between PRRs and other molecules on immune cells detecting C. albicans.*** *Dectin-1 is sequestered from active PRR domains by CD37, and clusters with TLR2/6, and Galectin-3 upon C. albicans detection, facilitating receptor collaboration (large arrows). Crosstalk with other molecules detecting C. albicans is suggested (dashed arrows). Interaction domains are hypothesized to be highly dynamic, varying in composition depending on the pathogen, cell type and activation status.*

### *Concluding remarks on the molecular network of fungal recognition*

Based on these studies, one can envisage a dynamic 3D-interaction-model, in which multiple receptors involved in sensing fungal pathogens can cluster together at the cell surface of innate immune cells (figure 1). Receptor compartmentalization provides immune cells with an efficient mechanism for regulated membrane-proximal signalling upon fungal detection. In the context of *C. albicans* recognition, extensive crosstalk between various PRRs has been shown, including the synergistic effects of Dectin-1, TLR2/6 and Galectin-3 on enhancing NF-κB mediated responses. In addition, negative regulatory circuits are in place, e.g. Dectin-1 interaction with tetraspanin CD37 was demonstrated to inhibit Dectin-1 signalling, possibly by sequestering Dectin-1 molecules away from activating PRR complexes.[50] Thus, receptor compartmentalization can act in both stimulatory and inhibitory manners to regulate PRR complexes. Furthermore, we anticipate that PRR complexes are highly dynamic and variable in composition, containing different PRRs and their adapter molecules, as well as non-PRR proteins. Depending on the fungal pathogen, the immune cell type and its activation status, the composition, dynamics and activity of the PRR complexes will be modulated. For example, Dectin-1 ligation with β-glucans reportedly induced NF-κB activation in bone marrow-derived DC, but not in bone marrow-derived macrophages. Furthermore, the Dectin-1/CARD9 signalling pathway is differentially activated in macrophages and DC. In conclusion, PRR crosstalk represents an important regulatory mechanism for the immunological response to pathogens *in vivo*. Future studies on PRR interactions are therefore necessary to increase further insight in this important process.

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# Chapter 3

# DC-SCRIPT: DC marker and regulator of TLR induced cytokine production

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## ABSTRACT

Dendritic cells (DCs) are the professional antigen presenting cells of the immune system that dictate the type and course of an immune response. Molecular understanding of DC biology is important for the design of DC based immunotherapies and optimal clinical applications in vaccination settings. Previously, we isolated and characterized the cDNA encoding DC-SCRIPT (dendritic cell–specific transcript, also known as ZNF366). DC-SCRIPT mRNA expression in the immune system was confined to DCs and was reported to be an early hallmark of DC differentiation. Here, we demonstrate IL-4 to be the dominant factor for DC-SCRIPT expression in human monocyte derived DCs (moDCs). In addition we show, for the first time, endogenous DC-SCRIPT protein expression in human DCs both *in vitro* and *in situ*. DC-SCRIPT protein is detected early upon differentiation of monocytes into DCs and is also present in multiple freshly isolated DC subsets. Maturation of DCs with TLR ligands further increased DC-SCRIPT mRNA expression, suggesting a role in DC maturation. Indeed, siRNA mediated knock-down of DC-SCRIPT affected the cytokine response upon TLR stimulation. These DCs displayed enhanced IL-10 and decreased IL-12 production, compared to *wild-type* DCs. Silencing of IL-10 in DC-SCRIPT knock-down DCs rescued IL-12 expression, suggesting a primary role for DC-SCRIPT in the regulation of IL-10 production.

## INTRODUCTION

Dendritic cells (DCs) are the professional antigen presenting cells of the immune system and play an essential role in the initiation and modulation of immune responses. DCs reside in the tissue in an immature state, and are capable of recognizing and capturing microbial antigens through specific receptors. Upon infection or inflammation, they undergo a complex process of maturation, where they change from antigen-capturing cells into antigen-presenting cells.[1] With the expression of co-stimulatory or co-inhibitory molecules and the secretion of pro- or anti-inflammatory cytokines, DCs generate either immunity or tolerance through T-lymphocyte stimulation.[2] The type of molecules that are expressed greatly depends on the activation status of the DC, and is affected by environmental stimuli.[3-5]

A broad range of DC subsets have been described, including the *in vitro* monocyte derived DCs (moDCs) and the *in vivo* blood derived myeloid and plasmacytoid DCs (mDCs and pDCs, respectively). Myeloid DCs act as sentinels in the periphery and have a specialized function depending on their location and Pattern Recognition Receptors (PRRs) expression profile. The cellular cues present at different locations, e.g. gut, skin or other organs, inflicted by local invading pathogens direct the mDCs towards a specific response. Plasmacytoid DCs are considered the front line of defense in anti-viral immunity as they rapidly produce massive amounts of type I interferon in response to viral infection and prime T cells against viral antigens.[6-8] *In vitro*, DCs can be generated from monocytes through stimulation with IL-4 and GM-CSF.[9] These cytokines trigger DC differentiation while inhibiting macrophage and osteoclast differentiation.[10-12]

The differentiation of the DC subsets from their precursors is a highly complex process. Genetic analyses have identified different transcription factors, including IRF4, RelB and PU.1, to be crucial in the development of specific DC subsets in lymphoid organs.[13-17] DC differentiation and maturation requires a complete change in the DC gene expression profile, mediated by the combinatorial effect of a few key transcription factors and chromatin re-organization.[18]

In 2006 we identified and characterized a new DC expressed transcription factor, termed dendritic cell–specific transcript (DC-SCRIPT; also known as ZNF366). DC-SCRIPT mRNA is present in all DC subsets tested so far, including moDCs, mDCs, pDCs and LCs (Langerhans cells). Interestingly, expression was not detected among other leukocyte populations,[19] suggesting an essential role of DC-SCRIPT in DC biology. Outside the immune system, DC-SCRIPT has also been detected in epithelial cells in the breast and in tumors derived thereof.[20,21] DC-SCRIPT is located on human chromosomes 5q13.2[22] and is encoded by an 8 kb messenger RNA. It is well conserved in evolution, with the human and mouse genes both located in syntenic chromosomal regions, sharing 80% amino acid sequence homology.[23] The protein consists of a proline rich region, 11 C2H2-type zinc fingers and an acidic region. In addition, it bears a functional CtBP1 motif and an LxxLL Nuclear Receptor (NR) interaction motif.[19,24] NRs are ligand-inducible transcription factors that bind specific DNA-regulatory response elements. NRs and their co-regulators have been described to play an important role in a wide variety of biological processes including immunobiology and cancer biology.[25-30] Moreover, we demonstrated that DC-SCRIPT is a unique modulator of NR function and a strong and independent prognostic marker in breast carcinoma.[20]

Thus far, the expression and function of DC-SCRIPT in DCs remains largely unknown. Here, we characterized the endogenous DC-SCRIPT protein expression dynamics in the *in vitro* monocyte derived DCs as well as in primary blood derived DCs and studied its functional role in DC maturation.

## MATERIALS & METHODS

*Generation of human DCs*

Human monocyte derived Dendritic Cells (moDCs) were generated from PBMCs as described previously.[31] Monocytes were derived from buffy coats. Plastic-adherent monocytes were cultured for 6 days in Phenol red free RPMI-1640 medium (Life Technologies, Breda, The Netherlands) supplemented with 1% ultra-glutamine (Cambrex, Wiesbaden, Germany), 0,5% antibiotic-antimycotic (Invitrogen, Breda, The Netherlands), 10% (v/v) FCS (Greiner, Kremsmuenster, Austria), IL-4 (300 U/ml), and GM-CSF (450 U/ml) both from cellgenix. During day 3 moDCs were supplemented with new IL-4 (300 U/ml) and GM-CSF (450 U/ml). Mature moDCs were generated from day 6 immature moDCs through 48 hour stimulation with 200 ng/ml LPS (InvivoGen, Toulouse, France). Human Myeloid Dendritic Cells (mDCs) were isolated from PBMCs using the CD1c (BDCA-1)$^+$ Dendritic Cell Isolation Kit (Miltenyi Biotec, Leiden, The Netherlands). Human Plasmacytoid Dendritic Cells (pDCs) were isolated from PBMCs using the CD304 (BDCA-4/Neuropilin-1)$^+$ MicroBead Kit (Miltenyi Biotec, Leiden, The Netherlands). Purity (>90%) of the freshly isolated mDCs and pDCs were ensured by FACS staining.

*RNA isolation and quantitative PCR*

Total RNA was isolated from cells using an RNA isolation kit (Zymo research). RNA quantity and purity were determined on a NanoDrop spectrophotometer. RNA was treated with DNase I (amplification grade; Invitrogen) and reverse-transcribed into cDNA by using random hexamers and Moloney murine leukemia virus reverse transcriptase (Invitrogen). mRNA levels for the genes of interest were determined with a CFX96 sequence detection system (Bio-Rad, Veenendaal, The Netherlands) with SYBR Green (Roche, Woerden, The Netherlands) as the fluorophore and gene specific oligonucleotide primers. The primers for DC-SCRIPT and PBGD were described previously.[20] Other used primers (forward, reverse): IL-12 (5’-ATGGCCCTGTGCCTTAGTAGT-3’, 5’-CGGTTCTTCAAGGGAGGATTTT-3’), IL-6 (5’-GCTATGAACTCCTTCTCCACAAGCG-3’, 5’-ATCCATCTTTTTCAGCCATCTTTGG-3’), TNF (5’-ATGAGCACTGAAAGCATGATCC-3’, 5’-GAGGGCTGATTAGAGAGAGGTC-3’), IL10 (5’-TCAAGGCGCATGTGAACTCC-3’, 5’-GATGTCAAACTCACTCATGGCT-3’). Reaction mixtures and program conditions were used that were recommended by the manufacturer (Bio-Rad). Quantitative PCR data were analyzed with the CFX Manager V1.6.541.1028 software (Bio-Rad) and checked for correct amplification and dissociation of the products. mRNA levels of the genes of interest were normalized to mRNA levels of the housekeeping gene porphobilinogen deaminase (*PBGD*) and were calculated according to the cycle threshold method.[32]

*Immunohistochemistry*

Snap-frozen tonsil specimens were obtained from the Department of Pathology, RUNMC St, Radboud and approved by the institutional ethics committee of the RUNMC. The specimens were embedded in OCT embedding matrix (CellPath, Newtown, UK) and sectioned in 5 µM thick tissue sections. The sections were placed on Superfrost slides (Thermo Scientific, Etten-Leur , the Netherlands), fixed with acetone, and incubated with 4 µg/mL goat anti-human DC-SCRIPT antibody (R&D Systems, Abingdon, UK), 4 µg/mL mouse anti-human DC-SIGN (AZN-D1), followed by incubation with a biotinylated horse anti-goat IgG or horse anti-mouse (Vector Laboratories), and signal development was performed using a Vectastain ABC-HRP Kit (Vector Laboratories, Burlingame, CA) and DAB (Sigma Aldrich, Zwijndrecht, the Netherlands). Isotype-matched goat IgG (R&D Systems) and mouse IgG1 (BD Bioscience) were used as controls. Sections were counterstained with hematoxylin to visualize the cell nuclei and analyzed by using a Leica DM LB microscope (Leica Microsystems B.V., Rijswijk, the Netherlands).

*Western blotting*

Cells were lysed in 1% SDS and 62,5mM Tris pH 6.8 and the protease inhibitors 2 µg/mL leupeptin (Sigma Aldrich), 2 µg/mL aprotinin (Roche), and 1 mM phenylmethylsulfonyl fluoride (Sigma Aldrich). Cell lysates were mixed with sample buffer containing 5% glycerol, 6% sodium dodecyl sulfate, 125 mM Tris–HCl (pH 6.8), 0.1 mg/mL bromophenol blue (Gebr . Schmid GmbH + Co, Freudenstadt, Germany), and 10%-mercaptoethanol (Sigma Aldrich); heated at 95°C for 5 minutes; and then cooled on ice. The proteins were resolved by electrophoresis on an 8% polyacrylamide gel (ratio of acrylamide to bisacrylamide, 37.5:1) and transferred overnight to Protran nitrocellulose transfer membranes (Schleicher and Schuell, 's-Hertogenbosch, the Netherlands) at 30 mA and 4°C. To block nonspecific protein binding, the membranes were incubated in 1% skimmed milk powder and 3% bovine serum albumin in PBST. The membranes were then incubated for 1 hr with 2,5 µg/ml goat anti-human DC-SCRIPT antibody (R&D Systems, Abingdon, UK), washed three times in PBST, and subsequently incubated for 1 hour with the secondary antibody IRDye 800CW donkey anti goat IgG (1:5000 dilution; Li-cor Biosciences, Bad Homburg, Germany ) to detect DC-SCRIPT. To detect actin, the membranes were incubated with a mouse anti-actin (1:20.000 dilution, Sigma clone AC-40), washed three times in PBST, and incubated for 1 hour with the secondary antibody Alexa Fluor 680 – conjugated Donkey-anti-mouse IgG (1:5000 dilution; Invitrogen). All membranes were then washed three times in PBST. After staining, the membranes were scanned by using an Odyssey Infrared Imaging System (Li-cor Biosciences) to visualize the proteins.

*Confocal Laser Scanning Microscopy*

Round ø 12 mm cover slides (Thermo Scientific, Braunschweig, Germany) were coated with Poly-L-Lysine (Sigma Aldrich). Immature and mature moDCs (day 6), mDCs and pDCs were seeded on cover slides (50.000 cell/slide) and adhered for 2 hours in serum free, phenol red free RPMI-1640 supplemented with 1% ultra-glutamine, 0,5% antibiotic-antimycotic, IL-4 (300 U/ml) and GM-CSF (450 U/ml). DCs were fixed using 1% paraformaldehyde extra pure DAC 1 (Merck, Haarlem, The Netherlands) in PBS for 15 min at RT. DCs were permeabilized with 100% ice cold Methanol (Boom, Meppel, The Netherlands) for 5 minutes at 4$^{o}$C, washed with PBS, blocked for 1 hour with 3% BSA (Roche) and 1% Normal Donkey Serum (Sigma Aldrich) in PBS, stained 1 hour with 2,5 µg/ml Goat-anti-human DC-SCRIPT (R&D Systems, Abingdon, UK) and 1 hour with 1/400 Alexa Fluor 488 Donkey anti-Goat IgG (Invitrogen). The nucleus was stained for 5 minutes with 0,3 µg/ml DAPI (Sigma Aldrich) or 1 µg/µl Propidium Iodide (ITK, Uithoorn, The Netherlands), washed with PBS and mounted on 76 x 26 mm microscope slide (Thermo Scientific) with mowiol + 2,5% azide (Calbiochem, San Diego, US). Confocal laser scanning microscopy (CLSM) was carried out with an Olympus FV1000 Confocal Laser Scanning Microscope with an Argon (457, 488, 515nm), and 405, 559 and 635 diode lasers at the Microscopic Imaging Facility of the Department of Cell Biology, Nijmegen Centre for Molecular Life Sciences, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands. The N/C ratio (mean Nuclear values/mean Cytoplasmic values) of DC-SCRIPT expression was calculated using a custom written quantitative image analysis algorithm in Fiji/ImageJ software (http://fiji.sc/).

*Goat-anti-DC-SCRIPT validation*

Immature moDCs were stained with 2,5 µg/ml goat-anti-human DC-SCRIPT or mouse-anti-human GR (Abcam, Cambridge, UK) for 40 minutes and 1 hour with 1/400 Alexa Fluor 488 Donkey-anti-goat or Goat-anti-mouse. Prior to staining the primary antibodies were supplemented with vehicle (2,9 µl 10 mM HCl + 2,9 µl 10mM NaOH) or 9,96 µg/ml recombinant human DC-SCRIPT (R&D Systems, Abingdon, UK).

*siRNA mediated knock-down*

For DC-SCRIPT silencing a 23 nucleotide Custom ZNF366 siRNA termed SC38 targeting the DC-SCRIPT gene at position 2349-2369 was used (Dharmacon, Lafayette, Colorado, US). For IL-10 silencing the ON-TARGETplus SMARTpool IL10 (Dharmacon) containing 4 different IL-10 targeting siRNA oligos each 21 nucleotides long was used. The irrelevant siRNA ON-TARGETplus Non-Targeting siRNA#1 (Dharmacon) was used as control. Cells were washed twice in PBS and once in OptiMEM without phenol red (Invitrogen). A total of 10 µg of siRNA was transferred to a 4-mm cuvette (Bio-Rad), and 10 × $10^6$ DCs were added in 200 µL of OptiMEM and incubated for 3 minutes before being pulsed with an exponential decay pulse at 300 V, 150 µF in a Genepulser Xcell (Bio-Rad) as described previously.[33] Immediately after electroporation, the cells were transferred to warm (37°C) DC culture medium without AA and supplemented with 1% ultra-glutamine, 0,5% antibiotic-antimycotic, 10% (v/v) FCS, IL-4 (300 U/ml), and GM-CSF (450 U/ml). Day 6 (72 hours after transfection) DCs were stimulated with vehicle, 200 ng/ml LPS, 4 µg/ml R848 (Axxora, Raamsdonkveer, The Netherlands) or 20 µg/ml PolyI:C (Sigma Aldrich) for 24 hours. RNA was isolated with the Quick-RNA MiniPrep kit (Zymoresearch). Total lysates were prepared 72 hours after transfection, lysing 50.000 cells in 50 µl 1% SDS lysis buffer containing 1% SDS and 62,5 mM TRIS pH 6,8 plus the protease inhibitors 2 µ g/mL leupeptin, 2 µg/mL aprotinin, and 1 mM phenylmethylsulfonyl fluoride.

*ELISA*

Cytokines were measured in the supernatants 24 hours after induction of maturation. IL-12p70 production was measured using a standard sandwich enzyme-linked immunosorbent assay (ELISA; Pierce Biotechnology, Etten-Leur, The Netherlands). IL-6 was measured using PeliPair human IL-6 ELISA kit (Sanquin, Amsterdam, The Netherlands), TNF was measured using Human TNF ELISA Set (BD Biosciences, Breda, The Netherlands), IL-10 was measured using Human IL-10 Module Set (Bender MedSystems, Vienna, Austria). Differences in cytokine production were assessed using *t* tests. Two-sided *P* values less than .05 were a priori considered to be statistically significant.

*Mixed Leukocyte Reaction*

DCs were electroporated at day 4 of differentiation with siSC or control siRNA and seeded in a 96-well plate (50000 cells/well). At day 7 DCs were stimulated with vehicle or 4 µg/ml R848 for 8 hours, after which the medium was replaced with fresh DC-medium. At day 8, 24 hours after R848 stimulation, PBLs were added to the DCs, in a ratio of 1:1, and co-cultured for 120 hours. After 4 days of co-culture cells were pulsed with [$^3$H]-thymidine for 15 hours, harvested, and [$^3$H]-thymidine incorporation was determined as a measure for T-cell proliferation. To analyze the T helper cell profile, supernatants were collected after 2 days of DC-PBLs coculture. Cytokine production in the supernatant were analyzed with a human Th1/Th2 Multiplex kit (eBioscience, Vienna, Austria) according to manufacturer's instructions.

## RESULTS

### *IL-4 induces DC-SCRIPT mRNA expression in monocytes*

Within the immune system, human DC-SCRIPT mRNA has been found to be preferentially expressed by DCs.[19] To obtain more insight into the expression characteristics of DC-SCRIPT, we investigated DC-SCRIPT mRNA expression during differentiation of monocytes into DCs. Hereto, adherent monocytes were cultured in the presence of IL-4,[34,35] GM-CSF or the combination of both cytokines. Cells were analyzed at different time points after start of differentiation. In the absence of cytokines (vehicle) essentially no DC-SCRIPT mRNA expression could be detected. In the presence of GM-CSF alone, only small amounts of DC-SCRIPT mRNA were discerned. Incubation with IL-4 and GM-CSF or IL-4 alone resulted in DC-SCRIPT mRNA expression within 2 hours after the start of stimulation, indicating IL-4 as the dominant factor for DC-SCRIPT induction. An increase in DC-SCRIPT mRNA levels was observed up to 8 hours after stimulation (figure 1A). At later time points, DC-SCRIPT mRNA levels decreased somewhat but remained stable from day 3 to day 8. DCs matured with LPS demonstrated an increase in mRNA expression levels (figure 1B), suggesting a role for DC-SCRIPT in DC maturation.

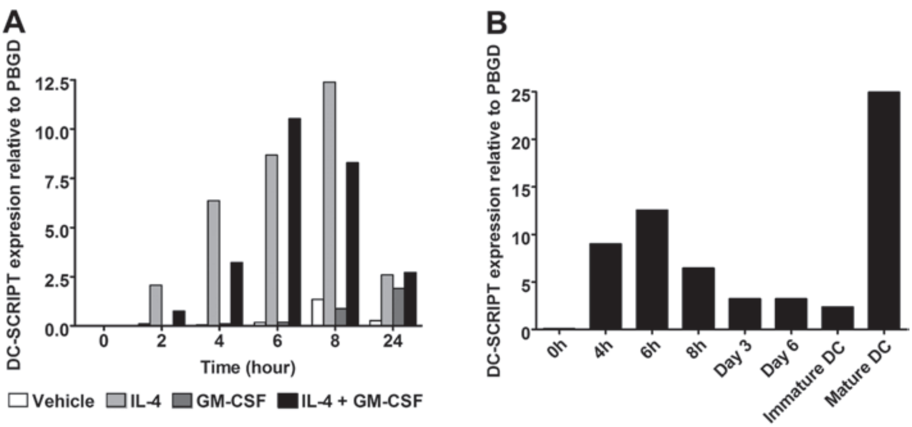


***Figure 1. DC-SCRIPT mRNA expression in moDCs***

*(A and B) DC-SCRIPT mRNA expression relative to PBGD mRNA in monocytes as determined by quantitative polymerase chain reaction. Monocytes were cultured in the presence of vehicle (white bars), IL-4 (light grey bars), GM-CSF (dark grey bars) or IL-4 and GM-CSF (black bars) and harvested at the indicated time points. Day 6 DCs were stimulated with vehicle or LPS for 48 hours to obtain immature and mature day 8 DCs, respectively. Representative data from 1 out of 3 donors.*

*DC-SCRIPT protein is expressed in different DC subsets*

To confirm endogenous DC-SCRIPT protein expression, cell lysates were prepared from monocytes at different time points after the onset of differentiation towards DCs. Within 4 hours, DC-SCRIPT protein expression could be observed. Protein levels steadily increased during differentiation to DCs and remained constant from day 6 onwards (Figure 2A). In line with its mRNA expression, DC-SCRIPT protein expression is also dependent on IL-4 (data not shown).

To prove that DC-SCRIPT protein is also present in freshly isolated blood mDCs and pDCs, cell lysates were prepared from purified mDCs and compared to total PBMC (peripheral blood mononuclear cells). DC-SCRIPT protein could not be detected in total PBMCs (figure 2B, lane 1), nor could it be detected in the mDC negative fraction (figure 2B, lane 2). However, in the mDC fraction DC-SCRIPT protein expression was readily observed (figure 2B, lane 3). Similarly, DC-SCRIPT protein was present in pDCs, albeit at lower levels compared to moDCs and mDCs (figure 2C, lane 3). No DC-SCRIPT expression could be detected in the total PBL (peripheral blood leukocyte) fraction (figure 2C, lane 1) and the pDC negative fraction (figure 2C, lane 2). These data show for the first time that DC-SCRIPT is endogenously expressed at protein level in freshly isolated mDCs and pDCs.

*DC-SCRIPT sub-cellular distribution varies among DC subsets*

The localization of endogenous DC-SCRIPT protein in the different subsets of DCs was investigated with confocal laser scanning microscopy (CLSM). Hereto, DCs were stained with anti-DC-SCRIPT antibodies recognizing the C-terminal part of DC-SCRIPT. The specificity of the antibody was validated by DC-SCRIPT peptide blocking experiments (supplemental figure S1).

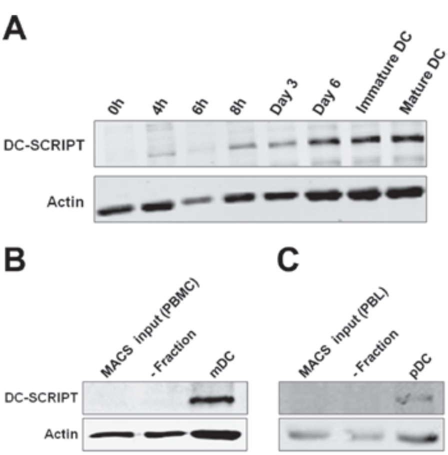


***Figure 2. DC-SCRIPT protein expression in moDCs, mDCs and pDCs***

*Proteins from cell lysates of the indicated cell fractions were subjected to immunoblotting with anti-DC-SCRIPT antibodies and anti-actin as loading control. (A) Cell lysates of moDCs harvested at the indicated time points. Day 6 DCs were stimulated with vehicle or LPS for 48 hours resulting in immature and mature day 8 DCs, respectively. (B and C) Cell lysates of the indicated fractions of an mDC (B) and pDC (C) isolation procedure.*

Our data show that DC-SCRIPT is predominantly localized in the nucleus of moDCs, and reveal that localization does not change upon maturation of the cells with LPS (figure 3A). Some DC-SCRIPT expression could be discerned in the cytoplasm. In fresh mDCs, DC-SCRIPT localization is also most pronounced in the nucleus of the cells. In pDCs, expression levels of DC-SCRIPT were apparently lower compared to mDC and moDC subsets. DC-SCRIPT staining could be found in both the cytoplasm and nucleus of pDCs, depending on the donor. Quantification of DC-SCRIPT expression in the nucleus and the cytoplasm was used to confirm localization differences between pDC donors, and between mDCs and pDCs. Between pDC donors the nucleus to cytoplasm (N/C) ratio varied between 1,1 and 2,4 (mean: 1,5 +/- 0,59) In contrast, mDCs displayed a N/C ratio of 2,5, whereas moDCs showed an average ratio of 3. The variation in N/C ratio within pDCs and mDCs from a single donor was minimal. These data show that the DC-SCRIPT localization is predominantly nuclear in moDCs and mDCs, while in pDCs a more pronounced cytoplasmic DC-SCRIPT staining is observed that varies between different pDC donors.

To further confirm DC-SCRIPT protein expression in DCs in immunological tissue, frozen tonsil sections were analyzed for DC-SCRIPT expression. The presence of DCs was confirmed by staining the consecutive section with the DC marker DC-SIGN (figure 3B). As expected, DCs with myeloid appearance were mainly present in the T-cell area in between the germinal centers, as shown by the DC-SIGN staining. The area in which DC-SCRIPT positive cells were found overlapped with the area containing DC-SIGN, a previously defined marker for myeloid DCs.[36] Furthermore, also DC-SCRIPT expression was observed in cells with myeloid DC morphology located in the T-cell area, further substantiating its protein expression in mDCs. In situ, DC-SCRIPT expression in the observed myeloid DCs appeared to be mostly confined to the nucleus. The low expression levels of DC-SCRIPT in pDCs, and their low abundance in lymph nodes, did not allow proper assessment of pDCs with this approach.

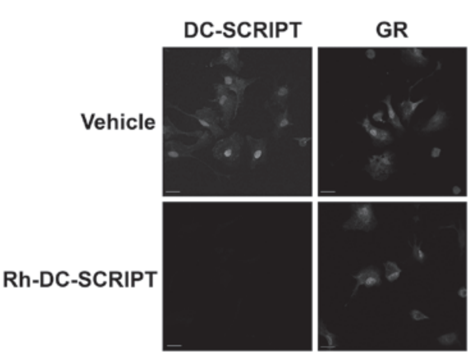


***Figure S1. Validation of the goat-anti-DC-SCRIPT antibody***

*CLSM validation of the anti-DC-SCRIPT antibody using a recombinant human DC-SCRIPT peptide (rh-DC-SCRIPT). This peptide represents the epitope for the anti-DC-SCRIPT antibody. Mouse-anti-GR antibody was used as control. Cells were stained with goat-anti-DC-SCRIPT or mouse-anti-GR pre-incubated with vehicle or rh-DC-SCRIPT.*

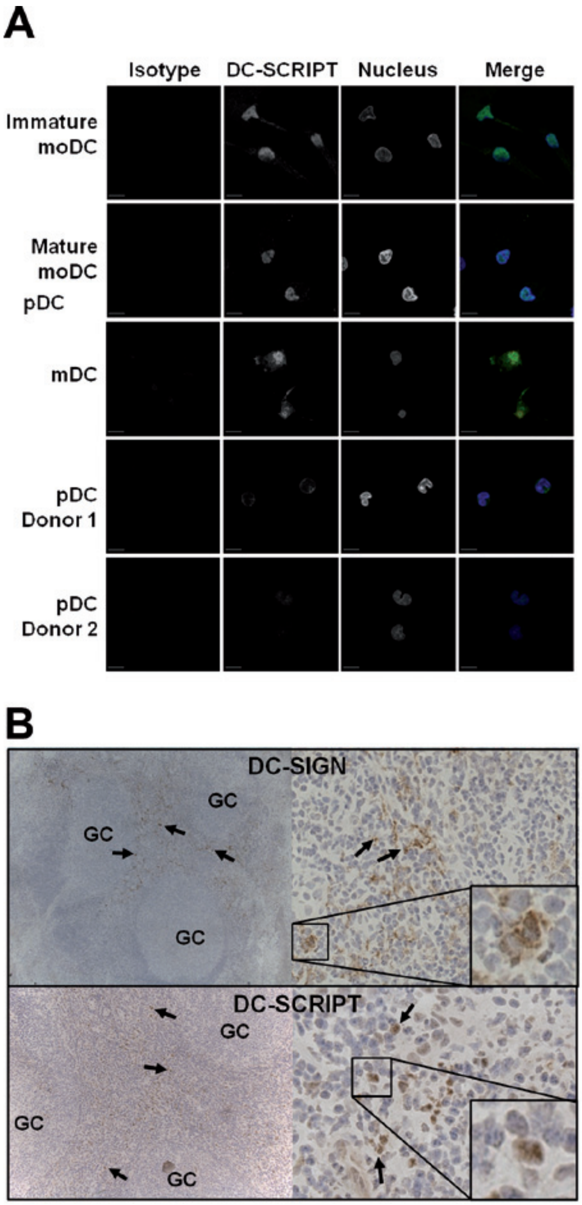


***Figure 3. DC-SCRIPT localization in different DC subsets***

*CLSM analysis of DC-SCRIPT expression in different DC subsets. DC-SCRIPT in immature and mature DCs (A) or pDCs was stained with a goat-anti-DC-SCRIPT (green), the nucleus was visualized by staining with DAPI (blue). DC-SCRIPT in myeloid DCs was stained with a goat-anti-DC-SCRIPT (green), the nucleus was visualized by staining with PI (red) The scale bar represents 10 µm. (B) Immunohistochemistry staining of DC-SIGN and DC-SCRIPT in frozen tonsil sections. Cells were counterstained with hematoxiline to visualize the nuclei.*

*DC-SCRIPT knock-down affects IL-10 secretion by moDCs*

DC-SCRIPT mRNA and protein is expressed during the complete lifecycle of moDCs, including in mature DCs. To investigate its function in DC maturation, DC-SCRIPT was silenced using a siRNA oligo (siSC) targeting the acidic region of the DC-SCRIPT gene at position 2349-2369. DCs treated with non-targeting siRNA oligos as well as non-treated DCs were used for comparison. Subsequently, immature DCs were stimulated for 24 hours with ligands for TLR4, -7/8 and -3, respectively LPS, R848 and PolyI:C. Our data demonstrate an efficient knock-down of DC-SCRIPT protein expression in siSC treated DCs at day 6 of differentiation prior to stimulation, but not control siRNA-treated and non-treated DCs (figure 4A).

The effect of DC-SCRIPT knock-down on DC maturation was examined by investigating cell surface maturation marker expression and cytokine secretion in the supernatant, at respectively 48 hours and 24 hours after stimulation. As expected, non-electroporated DCs markedly increased expression of both maturation markers CD80 and CD83 upon TLR stimulation (figure 4B). DCs electroporated with siSC or irrelevant siRNA (control) also enhanced CD80 and CD83 expression upon activation, at equal intensities. Relative to untreated DCs, electroporated DCs showed some disparity in CD83 expression upon LPS stimulation, possibly due to the variable maturation effects by LPS.

DC maturation was also qualified by analyzing cytokine secretion (figure 4C). Secretion of the pro-inflammatory cytokines IL-6 and TNF by non-stimulated DCs electroporated or not, could not be detected, in agreement with their immature status. Stimulation with TLR ligands differentially induced IL-6 and TNF secretion, with highest levels found upon R848 treatment, and lowest levels after PolyI:C stimulation. Both electroporated and not electroporated DCs secreted equal amounts of IL-6 and TNF. No significant effect of DC-SCRIPT silencing was detected relative to control siRNA treated and non-treated DCs. Variation after LPS treatment, was again detected between donors. In line with the absence of pro-inflammatory cytokine expression, also secretion of the anti-inflammatory cytokine IL-10 was minimal in all conditions in immature DCs. As expected, TLR mediated maturation did induce only a minimal amount of IL-10 expression in both siRNA control DCs and non-treated DCs. Surprisingly, DCs electroporated with siSC displayed a significant increase in IL-10 secretion after treatment with LPS or R848. Little or no effect of DC-SCRIPT silencing could be detected upon PolyI:C stimulation. Hence, these data demonstrate that DC-SCRIPT expression in DCs is important for repression of IL-10 secretion during TLR4 and -7/8 induced maturation.

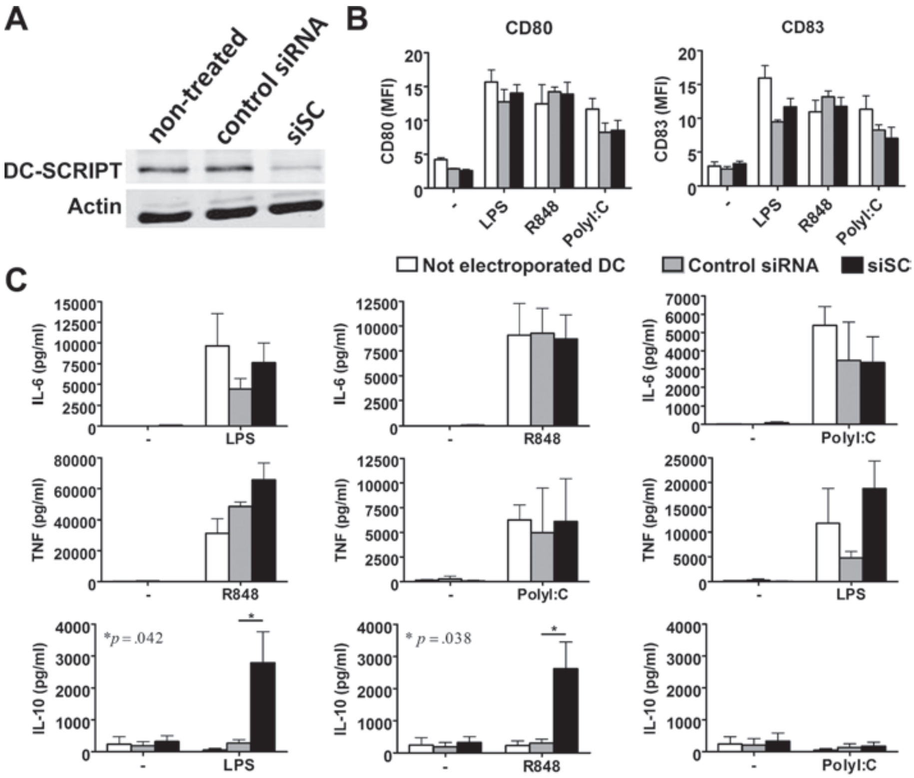


***Figure 4. Cytokine secretion by DC-SCRIPT knock-down moDCs***

*Day 4 moDCs were not electroporated (non-treated), or electroporated with control siRNA or siSC oligos. At day 6, vehicle, LPS, R848 or PolyI:C were used to mature the DCs. (A) DC-SCRIPT expression at day 6, prior to stimulation, was analyzed by western blot analysis. DC-SCRIPT was visualized by immunoblotting with anti-DC-SCRIPT and anti-actin as a loading control. Maturation of non-treated DCs (white bars), control siRNA treated DCs (grey bars) and siSC treated DCs (black bars) was analyzed by measuring maturation markers CD80 and CD83 by means of FACS (B) and secretion of IL-6, TNF and IL-10 in the supernatant by means of ELISA (C), respectively 48 hours and 24 hours after stimulation. Data from at least 3 donors. Error bars correspond to +/- SEM.*

### *Increased IL-10 secretion in siSC DCs impairs IL-12 secretion*

The anti-inflammatory cytokine IL-10 is known to impair DC maturation, including IL-12production.[2] To gain more insight into the kinetics of cytokine production in siRNA control and DC-SCRIPT knock-down DCs, cytokine mRNA and protein levels were monitored in time. To this end, IL-6, TNF, IL-12 and IL-10 mRNA and protein expression of control siRNA or siSC electroporated DCs was measured at 0-, 2-, 4-, 8-, 16- and 24 hours after R848 stimulation (figure 5). Both siSC and control siRNA treated cells demonstrated maximum mRNA expression between 2 and 16 hours for all cytokines, which decreased at later time points. Maximum levels of IL-6 and

TNF expression were detected 4 hours after stimulation, whereas IL-12p35 and IL-10 mRNA expression peaked at 8 hours. The protein expression of IL-6, TNF and IL-10 followed the mRNA expression kinetics, reaching maximum levels at later time points, after which expression remained relatively stable. When comparing control siRNA and siSC treated DCs, no effect was found for IL-6 or TNF secretion. In contrast, siSC and control siRNA DCs differed greatly in the expression of IL-10 and IL-12. In addition to the increase in IL-10 production, IL-12 production was significantly impaired at both the mRNA and the protein level in siSC DCs. The impaired IL-12 expression observed in siSC DCs was preceded by the increased IL-10 secretion, suggesting a role for IL-10 in reducing IL-12 levels in siSC DCs.

*IL-10 silencing rescues IL-12 secretion in DC-SCRIPT knock-down DCs*

In order to confirm the role of enhanced IL-10 secretion on the expression of IL-12, both DC-SCRIPT and IL-10 expression were silenced. To this end, DCs were electroporated with either control siRNA, siSC with control siRNA or siSC with IL-10 targeting siRNA (siIL-10).

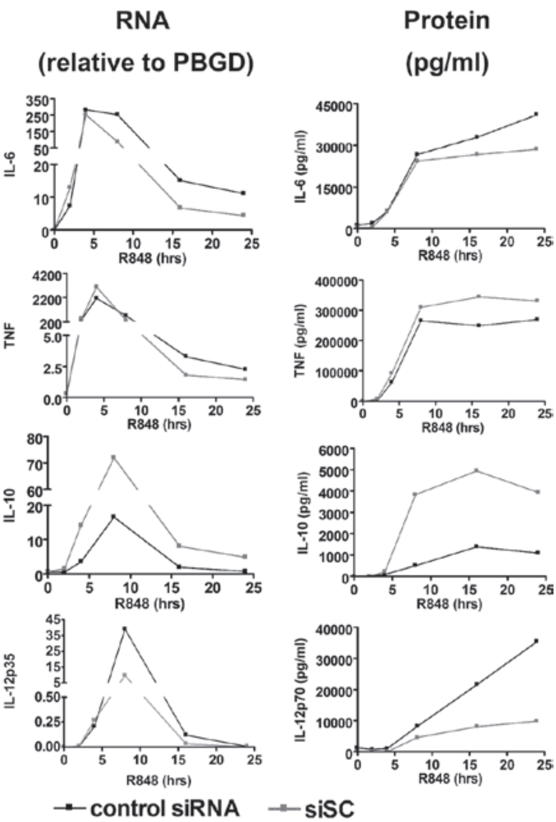


***Figure 5. Kinetics of cytokine expression***

*Day 4 moDCs were electroporated with control siRNA (black) or siSC oligos (grey). At day 6 DCs were stimulated with vehicle or R848. Messenger RNA and protein were measured at the indicated time points for IL-10, IL-12, IL-6 and TNF expression by means of Q-PCR and ELISA, respectively. Representative data from 1 out of 3 donors.*

Day 6 DCs were stimulated with R848 for 24h after which cytokine production was measured in the supernatant. As an additional control, untreated DCs stimulated with R848 received $10^6$ U/ml IL-10 or vehicle 4 hours after R848 stimulation, in order to mimic the enhanced IL-10 secretion in siSC DCs.

Again, efficient knock-down of DC-SCRIPT protein expression was detected in all conditions electroporated with siSC prior to stimulation (figure 6A). IL-10 protein expression in the supernatant was measured at both 8 hours (data not shown) and 24 hours after stimulation and demonstrated efficient IL-10 silencing at both time points. DC-SCRIPT knock-down DC again showed decreased IL-12 secretion upon R848 simulation. Likewise, addition of recombinant IL-10 to untreated DCs stimulated with R848 resulted in a significant (p=.011) reduction in IL-12 secretion (figure 6B). Silencing of both DC-SCRIPT and IL-10, however, resulted in normal IL-12 protein levels, confirming that IL-10 signaling mediates down-regulation of IL-12 secretion in siSC DCs (figure 6C). In line with previous results, no effect was detected on IL-6 and TNF secretion, suggesting that the enhanced IL-10 production specifically affects IL-12 expression in these cells.

*DC-SCRIPT knock-down impairs T-cell responses*

Next, we investigated the biological consequences of DC-SCRIPT silencing on DC-mediated T-cell responses in an allogeneic Mixed Leukocyte Reaction (MLR).

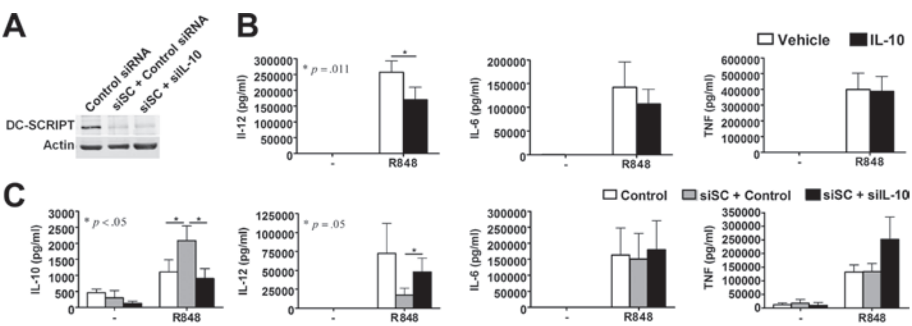


**Figure 6.** ***Cytokine secretion by moDCs with silenced DC-SCRIPT and IL-10 expression***

*Day 4 moDCs were electroporated with control siRNA, siSC + control siRNA or siSC + siIL-10 oligos. At day 6 DCs were stimulated with vehicle or R848. (A) Western blot analysis of DC-SCRIPT expression at day 6, prior to stimulation. DC-SCRIPT was visualized by immunoblotting with anti-DC-SCRIPT and anti-actin as a loading control. (B) Day 6 immature DCs were stimulated with R848, or not, and treated with vehicle or IL-10 4 hours after R848. IL-12, IL-6 and TNF secretion in the supernatant was measured 24 hours after R848 by means of ELISA. Data from 5 donors. Error bars correspond to +/- SEM. (C) Analysis of IL-10, IL-12, IL-6 and TNF secretion in the supernatant of control siRNA DCs (white bars), siSC + control siRNA DCs (grey bars) and siSC + siIL-10 DCs (black bars), 24 hours after stimulation, by means of ELISA. Data from at least 3 donors. Error bars correspond to +/- SEM.*

SiSC or control siRNA electroporated DCs were stimulated at day 7 with vehicle or R848 for 8 hours, after which the medium was replaced with fresh DC-medium. Twenty-four hours after stimulation, PBLs were added and both T-cell proliferation and cytokine secretion were determined as a measure of T-cell activation. As shown (figure 7A), T-cell proliferation was readily detected upon stimulation with control siRNA treated DCs and was significantly impaired after stimulation with siSC treated DCs. The effect of R848 stimulation on T cell proliferation was limited, possibly reflecting the overall immune activation at these allogeneic conditions.

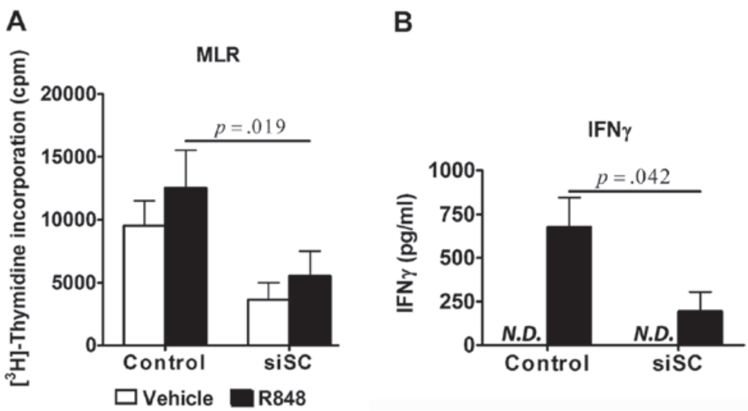


***Figure 7. DC-SCRIPT knock-down affects T-cell activation***

*Effect of DC-SCRIPT silencing on T-cell proliferation (A) and IFNγ secretion (B) in an allogeneic mixed leukocyte reaction (MLR). Day 4 moDCs were electroporated with control siRNA or siSC oligos. At day 7 DCs were stimulated with vehicle or R848 for 8 hours. Day 8 DCs were co-cultured with PBLs in a ratio of 1:1. Data from at least 3 donors. Error bars correspond to +/- SEM. Data indicated with N.D. (not detected) were below the detection limit.*

In contrast to T cell proliferation, IFNγ secretion by T-cells in these co-cultures was largely dependent on the presence of R848 (figure 7B). Strikingly, DC-SCRIPT silenced DCs showed an impaired capacity to induce IFNγ secretion by T-cells relative to control siRNA silenced DCs. No significant differences were detected for the pro-inflammatory cytokine levels of IL-6 and TNF between control and siSC treated DCs in these co-cultures (supplemental figure S2). Collectively, these data strengthen the finding that DC-SCRIPT plays an important role during DC maturation and the induction of T cell responses.

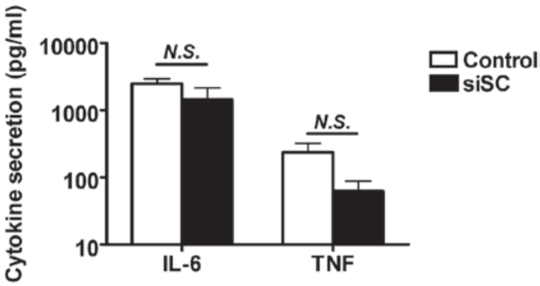


**Figure S2.** ***DC-SCRIPT knock-down does not affect IL-6 and TNF secretion in a MLR***
*Effect of DC-SCRIPT silencing on IL-6 and TNF secretion in an allogeneic mixed leukocyte reaction (MLR). Day 4 moDCs were electroporated with control siRNA or siSC oligos. At day 7 DCs were stimulated with vehicle (not shown) or R848 for 8 hours. Day 8 DCs were co-cultured with PBLs in a ratio of 1:1. Data from 3 donors. Error bars correspond to +/- SEM. Data indicated with N.S. (not significant) did not meet the criteria in the t test to be considered statistically significant.*

## DISCUSSION

Previously, we have isolated and characterized the cDNA encoding the transcription regulator DC-SCRIPT[19,23] that is preferentially expressed in DCs within the immune system. In the present study, DC-SCRIPT mRNA and protein expression were found to be induced early in DC differentiation and were dependent on IL-4.

Silencing of DC-SCRIPT expression affected DC maturation and induced IL-10 secretion in mature DCs, whichconsequently impaired IL-12 secretion by these cells. Furthermore, DC-SCRIPT silenced DCs were shown to have a significantly impaired capacity to induce T-cell proliferation and IFNγ responses. Hence, DC-SCRIPT appears to be an important factor in regulating DC maturation.

In human moDCs, DC-SCRIPT expression was dependent on the presence of IL-4. Control experiments demonstrated no DC-SCRIPT expression in PBLs upon IL-4 stimulation (data not shown). In addition, although *in vivo* studies previously demonstrated DC-SCRIPT expression in breast epithelial cells,[20] IL-4 stimulation of the DC-SCRIPT negative MCF-7 breast carcinoma cells did not induce DC-SCRIPT expression (data not shown). These data therefore suggest that the IL-4 mediated induction of DC-SCRIPT is related to the differentiation of monocytes to DCs. It is therefore important to further deduce the expression and function of DC-SCRIPT in the DC differentiation process itself.

Next to moDCs, DC-SCRIPT expression was readily detected in mDCs and pDCs. Previous reports demonstrated DC-SCRIPT mRNA expression in all DC subsets tested, including LCs, mDCs and pDCs.[19] Here we show, for the first time, endogenous DC-SCRIPT protein expression in both mDCs and pDCs. Moreover, *in vivo* DC-SCRIPT expression was observed in DCs present in T-cell areas of tonsil tissue. In IHC- and CLSM slides DC-SCRIPT is predominantly localized in the nucleus of moDCs and mDCs, concomitant with the presence of a nuclear localization motif. Interestingly, some expression was also detected in the cytoplasm of moDCs and mDCs. This was even more pronounced in pDCs, however, it must be noted that expression is lower and that variations between donors were observed for this subset. In addition, our recent work demonstrated a predominant cytoplasmic expression of DC-SCRIPT in breast epithelial cells.[20] This suggests that DC-SCRIPT might also have important functions outside the nucleus.

Further insight into the cytoplasmic expression of DC-SCRIPT might be gained from the NR biology. In a previous publication we have characterized DC-SCRIPT as a NR co-regulator.[20] NRs and many of their co-regulators are found both in the nucleus as well as in the cytoplasm. The type I NRs are classically sequestered in the cytoplasm, and translocate to the nucleus upon ligand binding, where they bind to specific DNA sequences.[26,37-40] NR function is controlled by NR co-regulators, such as N-CoR and SMRT. Recent evidence suggests shuttling of these co-regulators between the nucleus and cytoplasm, triggered by changes in signaling at the cell surface.[41] Further research is necessary to fully elucidate the pattern and dynamics of DC-SCRIPT expression in different DC subsets. Investigating the effects of various extracellular signals, such as NR ligands, will provide more insight into the DC-SCRIPT localization characteristics in DCs.

In addition, our data uncovered an important role for DC-SCRIPT in DC maturation. DCs with silenced DC-SCRIPT expression displayed enhanced IL-10 and decreased IL-12 cytokine production upon maturation. Silencing IL-10 expression rescued the IL-12 secretion in DC-SCRIPT knock-down DCs, suggesting that DC-SCRIPT primarily affects the expression of IL-10. No effect was detected on IL-6 and TNF secretion, or after PolyI:C maturation. Interestingly, TLR4 and -7/8 signal via the adaptor protein MyD88, whereas TLR3 activates TRIF mediated signaling,[42] suggesting that DC-SCRIPT primarily functions in the MyD88 pathway. Functionally, DC-SCRIPT silenced DCs are less capable of inducing T-cell proliferation and IFNγ secretion in a co-culture with allogeneic PBLs.

Unraveling the underlying molecular mechanisms by which DC-SCRIPT can regulate IL-10 expression in DCs will be an important next step. In DCs, IL-10 transcription is induced through NF-κB activation.[43] One way of enhanced and prolonged IL-10 transcription is NF-κB acetylation.[44] It would therefore be of great interest to see whether, and how DC-SCRIPT is involved in NF-κB acytylation. Furthermore, besides immunostimulatory DCs, future studies on DC-SCRIPT expression and function should also include tolerogenic DCs (tolDCs). These immunosuppressive DCs are known to secrete elevated levels of IL-10, while having diminished IL-12 secretion, thereby preventing T-cell proliferation.[45] Studying tolDCs is even more interesting as we have recently found that DC-SCRIPT also affects the function of the Glucocorticoid Receptor (GR) (Hontelez *et al.*, submitted). GR is well known for its central role in the generation of tolerogenic DCs, and known to induce IL-10 production. Moreover, the IL-10 promoter contains a Glucocorticoid Responsive Element (GRE), that could serve as a binding site for GR to stimulate IL-10 transcription.[46,47]

DCs are in the centre of the immune system, controlling the type and course of an immune response. They regulate both innate and adaptive immunity and serve as a bridge between both systems. Therefore, DCs are regularly used in immunotherapy. Detailed understanding of DC differentiation and maturation will allow for the generation of the best suitable DC for these therapies. Collectively, our data provide important insight in the DC biology, highlighting DC-SCRIPT as an essential factor in DC maturation.

## Acknowledgements

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# Chapter 4

# DC-SCRIPT regulates Glucocorticoid Receptor function and expression of its target GILZ in dendritic cells

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## ABSTRACT

Dendritic cells (DCs) play a central role in the immune system; they can induce immunity or tolerance depending on diverse factors in the DC environment. Pathogens, but also tissue damage, hormones and vitamins affect DC activation and maturation. In particular glucocorticoids (GCs) are known for their immunosuppressive effect on DCs, creating tolerogenic DCs (tolDCs). GCs activate the Type I Nuclear Receptor (NR) Glucocorticoid Receptor (GR), followed by induced expression of the transcription factor GILZ (Glucocorticoid Inducible Leucine Zipper). GILZ has been shown to be necessary and sufficient for GC induced tolDC generation. Recently, we have identified the dendritic cell specific transcript (DC-SCRIPT) as a NR co-regulator, suppressing type I steroid NRs ER (estrogen receptor) and PR (progesterone receptor). Here, we analyzed the effect of DC-SCRIPT on GR activity. We demonstrate that DC-SCRIPT co-exists with GR in protein complexes, and functions as a co-repressor of GR mediated transcription. Co-expression of DC-SCRIPT and GR is shown in human monocyte derived DCs, and DC-SCRIPT knock-down enhances GR dependent up-regulation of GILZ mRNA expression in DCs. This demonstrates that DC-SCRIPT serves an important role in regulating GR function in DCs, corepressing GR dependent up-regulation of the tolerance inducing transcription factor GILZ. These data imply that by controlling GR function and GILZ expression, DC-SCRIPT is potentially involved in the balance between tolerance and immunity.

## INTRODUCTION

Dendritic cell (DC) maturation occurs upon pathogen recognition and inflammation in the peripheral tissue, and involves up-regulation of co-stimulatory molecules and inflammatory cytokine secretion. Fully matured DCs migrate to T-cell areas in the lymph nodes, inducing T-cell activation.[1] In addition, DCs can also be activated to become tolerogenic, suppressing inflammation. Both human and murine studies demonstrated that this process involves impaired DC activation, which can be triggered by ligand dependent activation of the Glucocorticoid Receptor (GR) prior to pathogen recognition. Glucocorticoid (GC) treated DCs displayed suppressed MHC-I restricted antigen presentation,[2,3] impaired up-regulation of co-stimulatory molecules[4,5] and reduced secretion of pro-inflammatory cytokines. Secretion of immunosuppressive cytokines was enhanced in these cells.[5-7] Collectively, these effects result in altered DC mediated T-cell activation, with reduced Th1 responses and selective expansion of T regulatory cells.[8,9] This does not only apply to monocyte derived DCs (moDCs), also myeloid DCs (MDCs)[10] and plasmacytoid DCs (PDCs)[11] have been shown to become tolerogenic following GC exposure.

The immunosuppressive effects of GCs are typically mediated by GR, a Type I Nuclear Receptor. The ligand-free form of GR predominantly resides in the cytoplasm, complexed to chaperone proteins. Conformational changes through ligand binding release GR from the chaperone complex, allowing for nuclear translocation and transcription initiation.[12]

The human GR is encoded by a single gene, and is expressed in virtually all cell types. Various GR isoforms have been described, and tissue specific effects are currently attributed to variation in GR isoform expression.[13-15] GR pre-mRNA can be alternatively spliced generating the transcriptional active GRα and the repressor GRβ, differing in sequence only at the C-terminus ligand binding domain (LBD). In contrast to GRα, GRβ expression is confined to the nucleus, where it antagonizes GRα dependent gene transcription.[16] Additionally, translation reinitiation occurs at seven AUG-start sites at the mRNA 5’-end, generating 8 different GR polypeptides. These isoforms, termed GR-A, -B, -C1, -C2, -C3, -D1, -D2 and -D3, differ in length at the N-terminus and in glucocorticoid responsiveness, differentially affecting target gene expression. The GR-A, -B and -C isoforms are localized in the cytoplasm, translocating to the nucleus upon ligand binding where they initiate target gene transcription. In contrast, GR-D isoforms are, independent of ligand binding, confined to the nucleus, and have markedly lower transcriptional activity.[14]

Recently, we have identified DC-SCRIPT (DC-Specific transcript) as a transcription factor and a NR co-regulator in human breast and prostate carcinoma tissue. DC-

SCRIPT was found to be present in Type I and Type II NR protein complexes, repressing Type I NRs ER, PR or AR (androgen receptor) mediated transcription, while enhancing Type II NRs RARα/RXR, VDR/RXR or PPARγ/RXR function.[17-20] In the immune system DC-SCRIPT is specifically expressed in DCs, and in contrast to other DC markers, identifies all DC-subsets tested to date.[21,22] Interestingly, we have demonstrated an important role for DC-SCRIPT in TLR4 and TLR7/8 mediated DC maturation. DC-SCRIPT knock-down in moDCs increased secretion of the anti-inflammatory cytokine IL-10, which subsequently impaired both the production of the pro-inflammatory cytokine IL-12, as well as T-cell proliferation.[23] Here, we investigated the putative role of DC-SCRIPT in controlling the anti-inflammatory function of GR in moDCs. Our data show the presence of DC-SCRIPT in GR protein complexes, and demonstrate altered GR mediated transcription in the presence and absence of DC-SCRIPT. Our findings therefore suggest active regulation of GR function by DC-SCRIPT in DCs.

## MATERIAL & METHODS

*Cell lines*

Human embryonic kidney 293 (HEK293) cells were cultured in DMEM containing GlutaMAX (Invitrogen, Breda, the Netherlands) supplemented with 10% heat-inactivated FCS (Greiner Bio-one, Alphen a/d Rijn, the Netherlands), 1% nonessential amino acids (Invitrogen), and 0.5% antibiotic–antimycotic (Invitrogen). Human hepatocellular carcinoma Hep3B cells were cultured in IMDM (Invitrogen) supplemented with 10% heat-inactivated FCS and 0,5% antibiotic-antimycotic (Invitrogen).

*Generation of human Dendritic Cells*

Human moDCs were generated from PBMCs as described previously.[24] Monocytes were derived from buffy coats. Plastic-adherent monocytes were cultured for 6 days in DCs culture medium (Phenolred free RPMI-1640 medium (Life Technologies, Breda, The Netherlands) supplemented with 1% ultra-glutamine (Cambrex, Wiesbaden, Germany), 0,5% antibiotic-antimycotic (Invitrogen, Breda, The Netherlands), 10% (v/v) FCS (Greiner, Kremsmuenster, Austria), IL-4 (300 U/ml), and GM-CSF (450 U/ml) both from cellgenix). During day 3 moDCs were supplemented with new IL-4 (300 U/ml) and GM-CSF (450 U/ml). Mature moDCs were generated from day 6 immature moDCs through 24 hr stimulation with vehicle followed by 24 hour stimulation with 200 ng/ml LPS (InvivoGen, Toulouse, France). Tolerogenic moDCs were generated from day 6 immature moDCs through 24 hour stimulation with 100 nM Dexamethasone and subsequent 24 hours with 200 ng/ml LPS. DC maturation was ensured by FACS staining.

*Confocal Laser Scanning Microscopy*

Round ø 12 mm cover slides (Thermo Scientific, Braunschweig, Germany) were coated with Poly-L-Lysine (Sigma Aldrich, Zwijndrecht, the Netherlands). Immature, mature and tolerogenic moDCs were seeded on cover slides (50.000 cell/slide) and adhered for 2 hours in serum free, phenolred free RPMI-1640 supplemented with 1% ultra-glutamine, 0,5% antibiotic-antimycotic, IL-4 (300 U/ml) and GM-CSF (450 U/ml). DCs were stimulated for 1 hour with 100 nM Dexamethasone or vehicle. DCs were fixed using 1% paraformaldehyde extra pure DAC 1 (Merck, Haarlem, The Netherlands) in phosphate-buffered saline (PBS) for 15 min at RT. DCs were permeabilized with 100% ice cold Methanol (Boom, Meppel, The

Netherlands) for 5 minutes at 4°C, washed with PBS, blocked for 1 hour with 3% BSA (Roche, Woerden, The Netherlands) and 1% Normal Donkey Serum (Sigma Aldrich) in PBS, stained 1 hour with 2,5 μg/ml (moDCs) Goat-anti-human DC-SCRIPT (R&D Systems, Abingdon, UK) and 2,5 μg/ml (moDCs) Mouse-anti-human GR (Abcam, Cambridge, UK) and 1 hour with 1/400 Alexa Fluor 488 Donkey-anti-Goat IgG and 1/400 Alexa Fluor 647 Rabbit-anti-Mouse IgG (Invitrogen). The nucleus was stained 5 minutes with 0,3 μg/ml DAPI (Sigma Aldrich), washed with PBS and mounted on 76 x 26 mm microscope slide (Thermo Scientific) with mowiol + 2,5% azide (Calbiochem, San Diego, US). Confocal laser scanning microscopy (CLSM) was carried out with an Olympus FV1000 Confocal Laser Scanning Microscope with an Argon (457, 488, 515nm), and 405, 559 and 635 diode lasers at the Microscopic Imaging Facility of the Department of Cell Biology, Nijmegen Centre for Molecular Life Sciences, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands.

*Plasmids*

The nuclear receptor isoforms GR, GR-C3 and GR-D3 (National Center for Biotechnology Information accession number) (NM_001018077) were isolated and cloned into pHA-n3 (described previously in,[18] generating pHA-n3/GR, pHA-n3/GR-C3, pHA-n3/GR-D3). The vectors pCATCH and pCATCH-DCSCRIPT were described previously.[22] pCATCH-DCSCRIPT was used to generate pCATCH-DCSCRIPTΔCtBPdm and pCATCH-DCSCRIPTΔLxxLL. The transcription reporter plasmid pMMTV-luc containing the mouse mammary tumor virus (MMTV) promoter was kindly provided by Prof. Dr. H. Stunnenberg (Department of Molecular Biology, Nijmegen Centre for Molecular Life Sciences, Radboud University Nijmegen, Nijmegen, the Netherlands). pMMTV-luc was used to generate pMMTVΔ-luc and pMMTVΔNF-I/Oct1-luc. pGRE-luc (Stratagene, La Jolla, United States) was used to generate pHRE1-luc, pHRE2-luc, pHRE3-luc and pHRE4-luc.

*Co-Immunoprecipitation assay*

Hek293 cells were seeded in 10 cm culture dish ($6 \cdot 10^6$ cells per dish) 24 prior to transfection. Cells were cotransfected with 5 μg pCATCH-DC-SCRIPT or pCATCH (control) and 5μg pHA-n3/GRα or pHA-n3 (control) by using Metafectene transfection reagent (Biontex, Martinsried/Planegg, Germany) according to the manufacturer's protocol. Cells were lysed 24 hours after transfection, in immunoprecipitation assay buffer (50 mM NaCl, 1% Triton X-100, 0.1% sodium dodecyl sulfate, 50 mM Tris – HCl [pH 7.5], 5 mM EDTA, and 0.5% sodium-deoxycholate) containing the protease inhibitors 2 μg/mL leupeptin (Sigma Aldrich), 2 μg/mL aprotinin (Roche, Woerden, the Netherlands), and 1 mM phenylmethylsulfonyl fluoride (Sigma Aldrich). Cell lysates were used for immunoprecipitation (IP) of DC-SCRIPT using anti-DC-SCRIPT-coupled dynabeads (GE Healthcare, Hoevelaken, The Netherlands) according to the manufacturer's protocol. Isotype-coupled-dyna-beads were used as a control.

*Western Blotting*

Proteins were subjected to electrophoresis on an 8% 37.5:1 acryl/Bisacrylamide gel and transferred onto Protan nitrocellulose transfer membranes (Schleicher and Schuell) for 1 hour at 100 V at 4°C. Blots were blocked in 1%Elk/3%BSA in Phosphate-buffered saline with 0.01% Tween (PBST). DC-SCRIPT was detected with anti-DC-SCRIPT (R&D, 1,2 μg/ml) and IRDye 680CW donkey-anti-goat IgG (1:5000 Li-cor biosciences) as secondary antibody. GR was detected with a rat anti-HA (1:1000, 3F10, Roche) and IRDye 800CW goat-anti-rat IgG (1:5000 Li-cor biosciences), or Anti-Glucocorticoid Receptor antibody [3D5] (Abcam, 200 μg/µl, 1:80) and IRDye 680CW goat-anti-mouse IgG (1:5000 Li-cor biosciences)as secondary antibody. Blots were probed with a mouse-anti-β-actin (1:20.000, Roche Applied Science) or rabbit-anti-actin (Sigma Aldrich) and IRDye 680CW donkey-anti-mouse IgG or goat-anti-rabbit (1:5000 Li-cor biosciences) as secondary antibody, as loading control. After staining, the membranes were scanned using the Odyssey™ Infrared Imaging system visualize the labeled proteins.

*Luciferase transcription assay*

Hep3b cells were plated ($6 \cdot 10^4$) in 24-wells plates 8 hours before transfection and transfected using the Calcium Phosphate precipitation method (Invitrogen). HEK293 cells were plated at ($1 \cdot 10^5$) in 24-wells plates 24 hours before transfection and transfected using metafectene. Transfected cells were stimulated with 100 nM dexamethasone, 100 nM Prednisolone or vehicle for 24 h. Cells were harvested 24 hours after stimulation and cell lysates were analyzed for luminescence according to manufacturer's protocol (Dual-Luciferase® Reporter assay, Promega) using a Victor$^3$ luminometer (PerkinElmer). Relative light units (RLU) were calculated after correction for transfection efficiency based on the activity of the co-transfected pRL-SV40 (Promega). The data are expressed as the mean activity of at least four independent experiments +/-s.e.

*DC-SCRIPT knock-down*

Human moDCs day 4 were electroporated with a 23 nucleotide Custom ZNF366 siRNA termed siSC targeting the DC-SCRIPT gene at position 2349-2369 (Dharmacon, Lafayette, Colorado, US) or the irrelevant siRNA ON-TARGETplus Non-Targeting siRNA#1 (Dharmacon) termed control. Cells were washed twice in PBS and once in OptiMEM without phenol red (Invitrogen). A total of 10 µg of siRNA was transferred to a 4-mm cuvette (Bio-Rad), and $10 \times 10^6$ DCs were added in 200 µL of OptiMEM and incubated for 3 minutes before being pulsed with an exponential decay pulse at 300 V, 150 µF in a Genepulser Xcell (Bio-Rad) as described previously.[25] Immediately after electroporation, the cells were transferred to warm (37°C) DC culture medium without AA and supplemented with 1% ultra-glutamine, 0,5% antibiotic-antimycotic, 10% (v/v) FCS, IL-4 (300 U/ml), and GM-CSF (450 U/ml). Day 6 (72 hours after transfection) DCs were stimulated with vehicle or 100 nM RU-486 (Sigma Aldrich), for 1 hour and subsequently with vehicle or 100 nM Prednisolone for 24 hours. RNA was isolated with the Quick-RNA MiniPrep kit (Zymoresearch). Total lysates were prepared 72 hours after transfection, lysing 50000 cells in 50 µl 1% SDS lysis buffer containing 1% SDS and 62,5 mM TRIS pH 6,8 plus the protease inhibitors 2 µ g/mL leupeptin, 2 µ g/mL aprotinin, and 1 mM phenylmethylsulfonyl fluoride.

*RNA isolation and quantitative PCR*

Total RNA was isolated from cells using an RNA isolation kit (Zymo research).RNA quantity and purity were determined on a NanoDrop spectrophotometer. Total RNA was DNase-I (amplification grade; Invitrogen) treated and cDNA was synthesized using random hexamers and Moloney murine leukemia virus reverse transcriptase (Invitrogen). mRNA levels for the genes of interest were determined with a CFX96 sequence detection system (Bio-Rad, Veenendaal, The Netherlands) with SYBR Green (Roche, Woerden, The Netherlands) as the fluorophore and gene specific oligonucleotide primers. The primers for DC-SCRIPT and PBGD were described previously.[18] Other used primers (forward, reverse): GR (5'- CCATTGTCAAGAGGGAAGGAAAC-'3, 5'- ATGATTTCAGCTAACATCTCGGG-'3), GILZ (5'-AGAACCTCAATACCGACAAG-'3, 5'- CATCAGATGATTCTTCACCA-'3). Reaction mixtures and program conditions were used that were recommended by the manufacturer (Bio-rad). Quantitative PCR data were analyzed with CFX Manager V1.6.541.1028 software (Biorad) and checked for correct amplification and dissociation of the products. As a reference gene the housekeeping gene porphobilinogen deaminase (PBGD) was used. DC-SCRIPT and GILZ levels relative to PBGD were calculated calculated according to the cycle threshold method.[26] Differences in mRNA expression were assessed using *t* tests. Two-sided *P* values less than .05 were a priori for samples to be considered statistically significant.

# RESULTS

*DC-SCRIPT co-immunoprecipitates GR*

DC-SCRIPT was previously shown to be present in protein complexes containing type I and/or type II NRs using co-immunoprecipitation (co-IP) assays.[18] Whether DC-SCRIPT is also present in GR containing protein complexes is unknown. To investigate this, whole cell lysates were prepared from HEK293 cells co-transfected with expression vectors encoding DC-SCRIPT and GR or their controls. Immunoprecipitations (IPs) were performed on these lysates using goat-anti-DC-SCRIPT-coated beads or control goat-IgG-coated beads. Both the immunoprecipitated fractions and the total lysates were subjected to immunoblotting. The data show an effective IP of DC-SCRIPT using goat-anti-DC-SCRIPT antibody-coated beads, whereas unspecific binding to the control beads is minimal (figure 1). In addition, GR was effectively co-immunoprecipitated with goat-anti-DC-SCRIPT-coated beads from lysates of HEK-293 cells that were co-transfected with DC-SCRIPT and GR. No GR co-IP was observed when DC-SCRIPT was co-transfected with control HA only. These data demonstrate that DC-SCRIPT and GR can exist in the same protein complex. This occurs most likely through indirect interaction, as the co-IP of GR with DC-SCRIPT could only be demonstrated when using mild lysis conditions (data not shown).

*DC-SCRIPT represses GR function on MMTV*

Next, we investigated whether DC-SCRIPT affects transcriptional activity of GR by using luciferase reporter assays. Hep3B cells were co-transfected with DC-SCRIPT, GR or their controls and a reporter construct containing the MMTV promoter controlling luciferase expression (MMTV-luc).

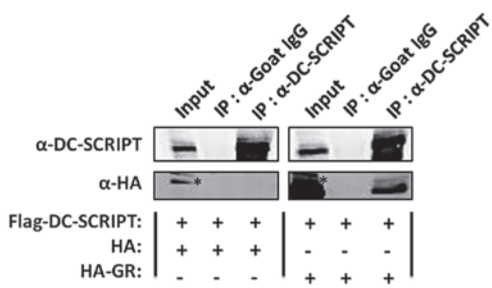


***Figure 1. Co-immunoprecipitation of GR with DC-SCRIPT***

*Association of DC-SCRIPT with the Glucocorticoid Receptor. Lysates prepared from HEK293 cells co-transfected with flag or flag-DC-SCRIPT and HA or HA-GR were subjected to immunoprecipitation using goat-IgG or goat-anti-DC-SCRIPT antibody-coated beads. Immunoblotting was performed on the IP proteins with goat-anti-DC-SCRIPT to detect DC-SCRIPT and rat-anti-HA to detect the Glucocorticoid Receptor. Data shown are from one of three experiments that produced the same result. * Indicates background band.*

In the MMTV promoter, four hormone responsive elements (HRE) have been identified that bind ligand activated GR, thereby inducing transcription of the luciferase gene.[27] Eighteen hours after transfection the cells were treated with vehicle, or the GR ligands Dexamethasone (Dex) or Prednisolone (Pred). Luciferase production was analyzed 24 h after stimulation.

Hep3B cells lack endogenous GR expression, therefore only background luciferase activity was detected after GR-ligand stimulation in the absence of ectopically expressed GR. Cells that were co-transfected with the GR expression vector did show luciferase activity in a GR ligand dependent manner. Both dexamethasone and prednisolone were able to induce luciferase production (figure 2A). Interestingly, increasing expression levels of DC-SCRIPT resulted in a dose- and ligand dependent repression of GR activity. Previously, DC-SCRIPT has been shown to directly interact with the global transcription co-repressor CtBP1 via its CtBP1 interaction motif.[22] In addition, DC-SCRIPT harbors a LxxLL motif, a motif known to facilitate interactions with NRs.[3] Next, we examined whether the CtBP1 and the NR binding (LxxLL) motifs within DC-SCRIPT were important for its repressive function on GR. We therefore mutated both CtBP domains or deleted the LxxLL motif. Figure 2B shows that both mutants are still able to strongly repress GR dependent luciferase production. Thus, DC-SCRIPT functions as a co-repressor of GR induced transcription on the MMTV promoter, independent of its CtBP1 binding- and LxxLL motif.

Efficient transcription initiation via the MMTV promoter requires the presence of the most distal HRE site (HRE1), the regulatory element *AA* upstream of HRE1[28] and binding of the transcription factors NF-I and Oct1.[27,29,30] To assess the requirement of these regulatory elements for DC-SCRIPT mediated co-repression, we deleted these sequence elements from the MMTV-luc reporter. In the MMTVΔ1-luc and MMTVΔ2-luc reporters the *AA* element alone, or both the HRE1 and the *AA* element are deleted, respectively, whereas the MMTVΔNF-I/Oct1-luc reporter lacks the NF-I and Oct1 binding sites[31] (figure 2C). The wild-type MMTV-luc reporter was used as positive control. Deletion of the *AA* element or the NF-I/Oct1 binding sites reduced GR dependent luciferase expression compared to the wt reporter, which was even further decreased upon deletion of both *AA* and HRE1 (figure 2D). On all MMTV reporters/mutants, DC-SCRIPT expression effectively repressed luciferase production. These data imply that that the repressive function of DC-SCRIPT does not depend on the HRE1 and the *AA* element, nor the NF-I and Oct1 binding sites.

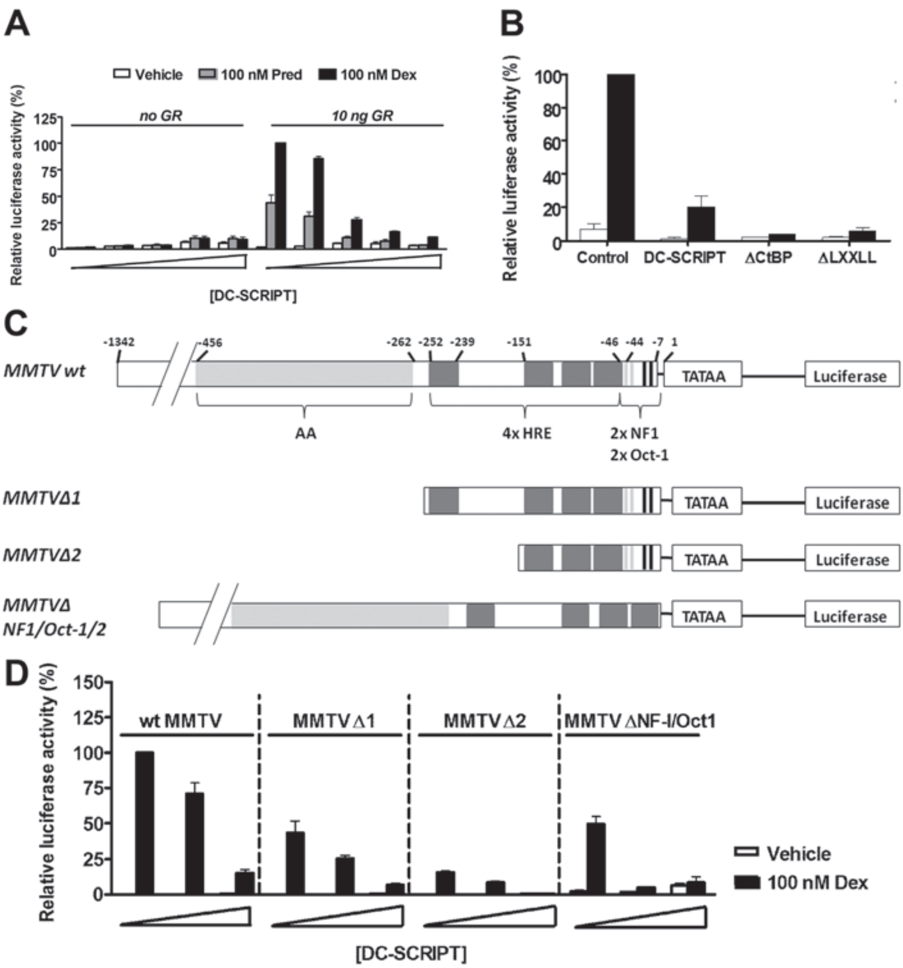


***Figure 2. Effect of DC-SCRIPT on GR mediated transcription***

*Luciferase reporter assay for GR mediated transcription. Hep3B cells were co-transfected with the firefly luciferase reporter plasmid MMTV-luc, the expression plasmid for GR and increasing amounts of the expression plasmid for wild-type DC-SCRIPT (A) or with mutated CtBP- or deleted LxxLL binding sites (B). Cells were stimulated with vehicle, 100 nM Dexamethasone or 100 nM Prednisolone for 24 h. Luciferase activity is displayed relative to luciferase production upon Dexamethasone stimulation in the presence of GR and absence of DC-SCRIPT. (C) Schematic representation of firefly luciferase reporters. Reporters consist of the MMTV promoter with 4 hormone responsive elements (HRE) in front of a TATA-box controlling luciferase gene expression. Three MMTV mutants have been created from the wild type (MMTVwt), deleting the upstream 3'-end including the AA domain (MMTVΔ1), the 3'-end and the most distal HRE (MMTVΔ2), or the binding sites for nuclear factor 1 (NF-I) and octamer transcription factor 1 (Oct1) (MMTVΔNF-I/Oct1). (D) Luciferase reporter assay for GR mediated transcription. Hep3B cells were co-transfected with the firefly luciferase reporter plasmids MMTVwt-luc, MMTVΔ1, MMTVΔ2 or MMTVΔNF-I/Oct1, the expression plasmid for GR and increasing amounts of the expression plasmid for DC-SCRIPT. Cells were stimulated with vehicle (white bars) or 100 nM Dexamethasone (black bars) for 24 h. Luciferase activity is displayed relative to luciferase production upon Dexamethasone stimulation in the presence of MMTVwt and in the absence of DC-SCRIPT. Data from at least 3 independent experiments. Error bars correspond to +/- SEM.*

*DC-SCRIPT represses transcriptional activity of GR isoforms*

GR has been shown to be translated into 8 different isoforms using alternative translational start sites (figure 3A, adapted from [15]). The GR expression vector includes all translational start sites, thus could express all GR isoforms. Indeed transfection of GR in HEK293 cells demonstrates expression of the full length GR-A, as well as the GR-B, -C, and, albeit at markedly lower levels, GR-D isoforms (figure 3A). To examine DC-SCRIPT function on different GR isoforms, we also cloned GR-C3 and GR-D3. Of note, the GR-C3 vector includes downstream ATGs, hence in addition to GR-C3, this vector could theoretically also express GR-D1, -D2, and -D3. However, only GR-C3 and GR-D1 could be detected (data not shown).

The effect of DC-SCRIPT on transcription initiation of the reporter construct MMTV-luc by these isoforms was tested using luciferase transcription assays. GR isoforms expressed by both GR and GR-C3 expression vectors equally induced luciferase production in a ligand dependent manner. Cells expressing the GR-D3 isoform, however, were less potent in inducing luciferase expression, displaying a more than 4-fold reduction in luciferase production. Increasing expression levels of DC-SCRIPT resulted in a dose- and ligand dependent repression of the transcriptional activity of all GR isoforms figure 3B). This indicates that the 1-336 amino acid N-terminal part of GR that was deleted in the GR-D3 expression vector is not required for the effect of DC-SCRIPT on GR dependent transcription.

*DC-SCRIPT and GR expression in immature, mature and tolerogenic DCs*

Next, we investigated DC-SCRIPT and GR protein expression by western blotting and confocal laser scanning microscopy (CLSM) in immature- (iDCs), mature- (mDCs) and tolerogenic DCs (tolDCs). Immature and mature DCs were obtained by stimulating immature moDCs with vehicle or 24 hours of LPS, respectively. Tolerogenic DCs were generated from immature moDCs through 24 hour stimulation with Dexamethasone and subsequent 24 hours with LPS. Western blot analysis of DC-SCRIPT and GR protein levels showed equal expression of both proteins in iDCs, mDCs and tolDCs (figure 4A). The translational isoforms GR-A, -B and -C, but not GR-D which lacks the epitope for the anti-GR antibody, could be detected in all conditions.

CLSM analysis revealed co-expression of DC-SCRIPT and GR protein in all three DC types tested. Expression of both proteins was observed predominantly in the nucleus, no major changes were detected between iDCs, mDCs and tolDCs (figure 4B). Co-localization between DC-SCRIPT and GR could be discerned but areas containing either GR or DC-SCRIPT were at least as abundant. As expected, nuclear expression of GR protein was increased upon 1 hour dexamethasone stimulation. In contrast, DC-SCRIPT localization was unaffected by dexamethasone treatment. Hence these data clearly demonstrate nuclear- and, to a lesser extent, cytoplasmic co-expression and partial co-localization of DC-SCRIPT and GR in moDCs.

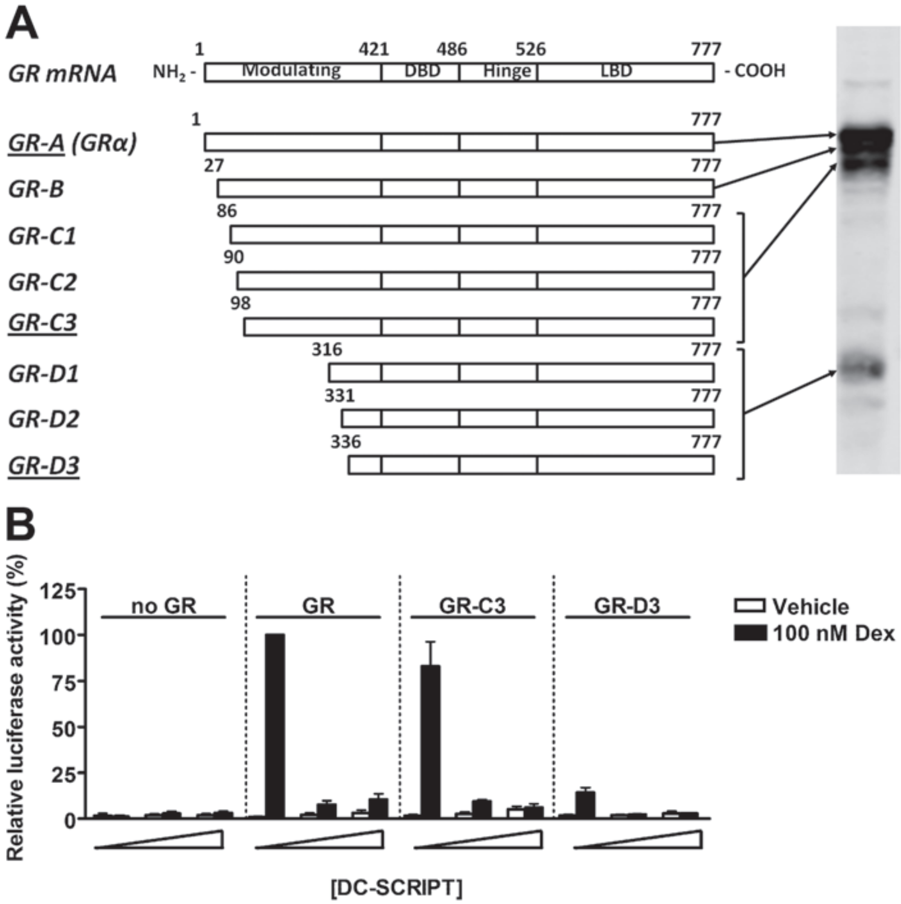


***Figure 3. Effect of DC-SCRIPT on translational isoforms of GR***

*(A) Translational isoforms of GR. One GR mRNA generates 9 isoforms by means of alternative translation initiation. Numbers indicate N- and C-terminal residue of each GR isoform. Different domains are indicated: the modulation domain contains the transactivation sequence, and is subject to phosphorylation and SUMOylation affecting protein interactions; the DNA binding domain (DBD); the hinge region and the ligand binding domain (LBD) (adapted from [15]). Expression plasmids have been created for underscored isoforms. The western blot shows the GR-A, -B, -C and -D isoforms visualized with rat-anti-HA in GR-HA transfected HEK293 cells . (B) Luciferase reporter assay for GR-A, GR-C and GR-D mediated transcription. Hep3B cells were co-transfected with the firefly luciferase reporter plasmid MMTV-luc, expression plasmids for GR-A, GR-C or GR-D and increasing amounts of the expression plasmid for DC-SCRIPT. Cells were stimulated with vehicle (white bars) or 100 nM Dexamethasone (black bars) for 24 h. Luciferase activity is displayed relative to luciferase production upon Dexamethasone stimulation in the presence of GR-A and in the absence of DC-SCRIPT. Data from at least 3 independent experiments. Error bars correspond to +/- SEM.*

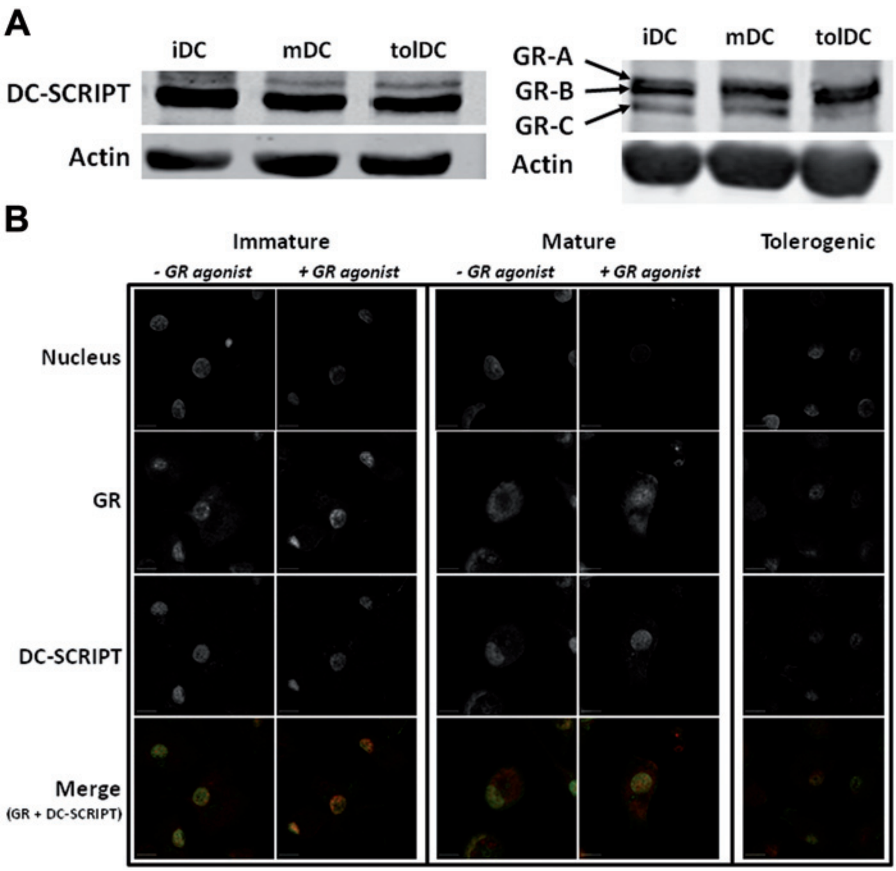


***Figure 4. DC-SCRIPT and GR expression in iDCs, mDCs and tolDCs***

*(A) Proteins from iDC, mDC and tolDC lysates were subjected to immunoblotting with anti-DC-SCRIPT or anti-GR antibodies and anti-actin as loading control. (B) CLSM analysis of DC-SCRIPT and GR expression in iDCs, mDCs and tolDCs. DC-SCRIPT was visualized with goat-anti-DC-SCRIPT (green), GR with mouse-anti-GR (red) and the nucleus with DAPI (not in merge). Immature and mature DCs were stimulated with vehicle or 100 nM Dexamethasone for 1 hour. Representative data from 1 out of 3 donors.*

### *Knock-down of DC-SCRIPT enhances GILZ expression in moDCs*

To determine the physiological relevance of the interaction between DC-SCRIPT and GR, DC-SCRIPT expression was silenced in moDCs using siRNA electroporation. As DC-SCRIPT was demonstrated to repress GR mediated transcription on the MMTV promoter, we hypothesized a similar effect on the endogenous GR target GILZ in moDCs. DC-SCRIPT knock-down was therefore expected to enhance GR dependent transcription, which could be detected by an increase in GILZ expression.

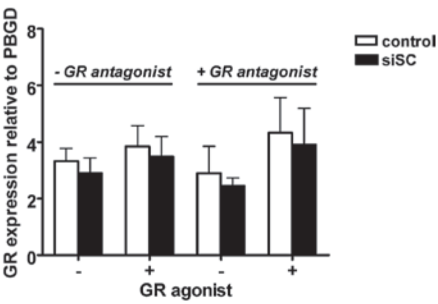


***Figure S1. DC-SCRIPT knock-down does not affect GR expression in moDCs***

*Day 4 human monocyte derived DCs were electorporated with control non-targeting siRNA (control, white bars) or DC-SCRIPT siRNA (siSC, black bars) and differentiated to day 6 immature DCs. At day 6 DCs were stimulated with vehicle or 100 nM RU-486 for 1hr prior to 24 h stimulation with vehicle or 100 nM Prednisolone. GR mRNA expression is shown relative to PBGD in mRNA lysates from day 7 DCs. Data from 3 donors. Error bars correspond to +/- SEM.*

Human moDCs were electroporated at day 4 with an DC-SCRIPT specific siRNA oligo (siSC) or an irrelevant control siRNA oligo. DC-SCRIPT protein expression was markedly reduced in siSC treated DCs 48- and 72 hours after electroporation (figure 5A), whereas GR expression remained unaltered (supplemental data S1). At day 6 moDCs were treated with vehicle or the GR antagonist RU-486 for 1 hour and subsequently stimulated with vehicle or the GR agonist prednisolone for 24 hours. After stimulation GILZ mRNA levels were measured. As expected, little or no GILZ mRNA expression was detected in vehicle treated control DCs. Stimulation with prednisolone resulted in an upregulation of GILZ expression levels in control DCs (figure 5B) which could be effectively blocked by inhibiting GR activation with the GR antagonist RU-486. Strikingly, vehicle treated siSC DCs already demonstrated a significant (p=.05) increase in GILZ expression compared to control DCs.

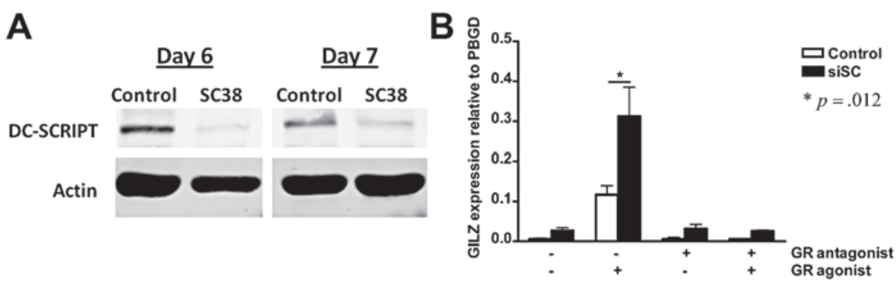


***Figure 5. DC-SCRIPT knock-down in moDCs***

*Day 4 human monocyte derived DCs were electorporated with control non-targeting siRNA (control, white bars) or DC-SCRIPT siRNA (siSC, black bars) and differentiated to day 6 immature DCs. At day 6 DCs were stimulated with vehicle or 100 nM RU-486 for 1hr prior to 24 h stimulation with vehicle or 100 nM Prednisolone. (A) DC-SCRIPT protein expression in protein lysates from day 6 and day 7 DCs. (B) GILZ mRNA expression relative to PBGD in mRNA lysates from day 7 DCs. Data from 6 donors. Error bars correspond to +/- SEM.*

This upregulation appears to be GR independent, as it is also detected in the presence of the GR antagonist RU-486. In the absence of the GR antagonist, treatment with prednisolone further increased GILZ expression in DC-SCRIPT knock down DC, with relative GILZ mRNA levels being significantly higher than in control siRNA treated DCs (p=.01). These data thus indicate DC-SCRIPT to function as co-repressor of GR mediated transcription of GILZ in DCs. Blocking GR activation with the GR antagonist abolished GILZ up-regulation in DC-SCRIPT knock-down DCs, confirming that this was indeed dependent on GR activation. These results indicate that DC-SCRIPT affects GR activity in DCs, and represses expression of the endogenous GR target gene GILZ, a transcription factor that has been associated with the generation of tolerogenic DCs.

## DISCUSSION

The present study demonstrates that the NR co-regulator DC-SCRIPT modulates GR function in human moDCs. In cell-lines, DC-SCRIPT was present in GR containing protein complexes, and GR mediated transcription was found to be repressed by DC-SCRIPT. Human moDCs co-express DC-SCRIPT and GR in the nucleus and cytoplasm, and DC-SCRIPT silencing resulted in enhanced expression of the GR target gene GILZ, a transcription factor that is instrumental for the generation of tolDCs.

Co-presence of DC-SCRIPT and GR in protein complexes could be demonstrated, and is in line with our previous findings, suggesting that DC-SCRIPT interacts with NRs via other proteins present in NR protein complexes. DC-SCRIPT has been shown to bind the transcription co-repressors RIP140 (receptor-interacting protein 140) and CtBP1, as well as histone de-acetylase (HDAC) 1, -3 and -6, all known to be present in NR protein complexes.[3,31,32] The absence of a direct contact between DC-SCRIPT and GR is also consistent with the finding that deletion of the NR interaction motif LxxLL and the CtBP1 interaction motif in the acidic domain of DC-SCRIPT did not affect its repressive function on GR transcription. This implies that interaction with CtBP1 and the presence of the LxxLL motif is not required for the repressive effect of DC-SCRIPT.

Co-expression of DC-SCRIPT and GR in DCs was demonstrated with CLSM, albeit areas containing either DC-SCRIPT or GR were equally abundant. Ligand dependent GR translocation was observed in iDCs. However, in the absence of its ligand, GR was also detected in the nucleus of iDCs. The nuclear GR expression most likely reflects the expression of GRβ isoforms, known to have restricted nuclear localization.[16] DC-SCRIPT expression was found to be mostly nuclear, in line with previous localization studies and its function as a transcriptional (co)factor.[32-34]

Within DCs, DC-SCRIPT was shown to co-regulate GR function affecting expression of the well known GR target gene GILZ. Knock-down of DC-SCRIPT expression markedly increased GILZ expression upon GR activation, implicating co-repression of GR by DC-SCRIPT on the GILZ promoter in DCs. GILZ functions as an effector protein of GR activation in DCs, mediating the anti-inflammatory action of GR. It has previously been shown that the immunosuppressive effect of GR can be reproduced in the absence of GR ligand by GILZ overexpression, whereas GILZ silencing abrogates the GR ligand effect.[34] GILZ has been shown to interact with STAT, NF-κB, AP-1, 14-3-3, Raf-1 and Ras proteins, all of which lead to inhibition of inflammation.[35] Hence, by regulating GR activity and GILZ expression, DC-SCRIPT may represent an important factor in DC biology, programming DCs towards immunity or tolerance. In line with this, we have recently demonstrated an important role for DC-SCRIPT in the repression of IL-10 secretion,[33] an anti-inflammatory cytokine known to be produced in large amounts by tolDCs.[36] In this study, DC-SCRIPT knock-down in immature moDCs significantly increased IL-10 expression levels upon TLR4 and -7/8 mediated maturation. Elevated IL-10 levels subsequently impaired IL-12 secretion and T-cell proliferation, suggesting that DC-SCRIPT knock-down skews DCs towards tolerance.

In addition to GILZ, we also investigated the expression of other genes known to be induced by ligand dependent GR activation in other cells. However, identification of these other GR targets in DC and whether they are affected by DC-SCRIPT proved to be difficult (data not shown). For eight out of ten tested target genes no GR-ligand dependent upregulation in moDCs could be detected. This could be due to cell-type specific differences between DCs and other cells, like differences in the expression and function of the repressive GRα-D and GRβ isoforms. Increased expression upon GR activation was only detected for FKBP5 (51 kDa FK506-binding protein 5) and PTX3 (pentraxin 3), however, induction levels were low and DC-SCRIPT silencing did not affect expression of these genes (data not shown). One major reason could be the HRE sequence or the context of this sequence in the promoter of these genes. Meijsing *et. al* (2009) recently demonstrated that a single nucleotide change in the GRE sequence influences GR binding affinity and conformation, affecting co-factor recruitment and transcription activation.[37] Similarly, the binding properties of DC-SCRIPT might differ depending on GR conformation and affect the binding of other co-factors. Furthermore, adjacent binding sites of other transcription factors can also affect GR mediated transcription and possibly DC-SCRIPT function.[17] In line with this, we have preliminary data that suggests that DC-SCRIPT can also activate GR dependent transcription, depending on the promoter context (data not shown). As soon as a suitable DC-SCRIPT antibodies for Chromatin-ImmunoPrecipitation (ChIP) become available, it would be extremely interesting to perform ChIP-sequencing studies and expression arrays, to further investigate gene regulation specifically in

DCs with respect to DC-SCRIPT and GR activation and expression.

Finally, besides GR, also other NRs are known to function in DC biology, affecting either differentiation, maturation, or both. The human NR superfamily contains 48 members, of which 20 NRs have been described to be expressed in monocyte derived DCs.[38] Since we have recently indentified DC-SCRIPT as a NR co-regulator in breast-[18] and prostate carcinoma,[20] affecting both type I and type II NR function, it is tempting to speculate about a role for DC-SCRIPT as NR co-regulator in DC biology. It would therefore be interesting to study target gene expression of various NRs after DC-SCRIPT knock-down in DCs.

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# Chapter 5

## DC-SCRIPT: nuclear receptor modulation and prognostic significance in primary breast cancer

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## ABSTRACT

*Background* Nuclear receptors, including estrogen receptor (ER), progesterone receptor (PR-B), peroxisome proliferator-activated receptor gamma (PPARγ), and retinoic acid receptor alpha (RARα), have been implicated in breast cancer etiology and progression. We investigated the role of dendritic cell-specific transcript (DC-SCRIPT) as coregulator of these nuclear receptors and as a prognostic factor in breast cancer.

*Methods* The effect of DC-SCRIPT on the transcriptional activity of nuclear receptors was assessed by luciferase reporter assays. DC-SCRIPT expression in normal and tumor breast tissue was analyzed by PCR assays and immunohistochemistry. The prognostic value of tumor DC-SCRIPT mRNA expression was assessed in three independent cohorts of breast cancer patients: a discovery group (n = 47) and a validation group (n = 97) (neither of which had received systemic adjuvant therapy), and a second tamoxifen-treated validation group (n = 68). Univariate and multivariable Cox proportional hazards model analyses were performed.

*Results* DC-SCRIPT suppressed ER- and PR-mediated transcription in a ligand-dependent fashion, whereas it enhanced the RARα- and PPARγ-mediated transcription. In breast tissue samples, DC-SCRIPT mRNA was expressed at lower levels in tumor than in corresponding normal tissues ($P$ = .010). Patients with high tumor DC-SCRIPT mRNA levels (66%) had a longer disease-free interval than those with a low DC-SCRIPT mRNA level (34%) (hazard ratio [HR] of recurrence for high vs low DC-SCRIPT level = 0.23, 95% confidence interval [CI] = 0.06 to 0.93, $P$ = .039), which was confirmed in the validation group (HR of recurrence = 0.50, 95% CI = 0.26 to 0.95, $P$ = .034). This prognostic value was confined to patients with ER- and/or PR-positive tumors (discovery group: HR of recurrence = 0.16, 95% CI = 0.03 to 0.89, $P$ = .030; validation group: HR of recurrence = 0.42, 95% CI = 0.19 to 0.91, $P$ = .028), and was also observed in the second validation group (HR = 0.46, 95% CI = 0.22 to 0.97, $P$ = .040). DC-SCRIPT was an independent prognostic factor after correction for tumor size, lymph node status, and adjuvant therapy (n = 145; HR = 0.50, 95% CI = 0.29 to 0.85, $P$ = .010).

*Conclusions* DC-SCRIPT is a key regulator of nuclear receptor activity that has prognostic value in breast cancer.

## INTRODUCTION

Nuclear receptors form a unique class of phylogenetically conserved transcriptional regulators.[1] They execute a transcriptional program upon binding to a ligand. Ligands for the nuclear receptor family of proteins vary from hormones to vitamins and metabolic products. In accordance with the wide variety of ligands, nuclear receptors are key regulators in a diversity of physiological functions, including development, metabolism, cell differentiation, and immune responses.[2] Malfunction of nuclear receptors has been associated with diseases such as diabetes, chronic inflammatory diseases, and cancer.[2-4] Clinically, a connection between hormone-dependent nuclear receptor function and breast cancer development has long been recognized. Most research has focused on the expression and function of two nuclear receptors, estrogen receptor-α (ER) and progesterone receptor (PR), which are preserved or increased in approximately 70% of breast tumors.[5] Estrogens, the ligands for the ER, are often associated with the initiation and progression of breast cancer,[6] and are well known for their proliferative effect on breast cancer cells.[7-9] Anti-estrogen therapy with tamoxifen has been applied successfully in the treatment of breast cancer patients.[5] In line with the importance of the ER and PR in breast cancer, the expression of transcriptional co-regulators of ER and PR are also of prognostic significance in breast cancer.[10,11] For example, the genes encoding nuclear receptor coactivator 3 (NCOA3; also known as *AIB1*) and nuclear receptor co-repressor 2 (*NCOR2*; also known as *SMRT*) have been shown to serve as a tumor suppressor gene and an oncogene for breast cancer, respectively.[12-17]

Another class of nuclear receptors, the retinoid X receptor alpha (RXRα) heterodimers (ie, RXRα–retinoic acid receptor alpha [RARα] and RXRα–peroxisome proliferator-activated receptor gamma [PPARγ]), have recently been implicated in breast cancer. For example, whereas the presence of ER and PR is associated with breast tumor development and breast tumor cell proliferation, RARα and PPARγ play a predominantly antitumorigenic role in human breast cancer by inhibiting cell growth and inducing apoptosis.[7,18] These properties imply that an imbalance in the activity of nuclear receptors may contribute to the development and progression of breast cancer. How the activity of the nuclear receptor repertoire in cells is regulated and which factors determine the response of the nuclear receptor repertoire to environments in which multiple nuclear receptor ligands are present are still open questions.

**CONTEXT AND CAVEATS**

**Prior knowledge** An imbalance in the transcriptional activities of estrogen receptor (ER), progesterone receptor (PR-B), peroxisome proliferator-activated receptor gamma (PPARγ), and retinoic acid receptor alpha (RARα) may contribute to the development and progression of breast cancer, but how the activities of these nuclear receptors are regulated is unclear.

**Study design** Luciferase reporter assays were used to examine the role of the novel transcriptional repressor dendritic cell-specific transcript (DC-SCRIPT) as a coregulator of ER, PR-B, PPARγ, and RARα in human cancer cells. DC-SCRIPT expression in normal and tumor tissue from breast cancer patients was analyzed by polymerase chain reaction assays and immunohistochemistry, and the prognostic value of tumor DC-SCRIPT mRNA expression was assessed in three independent cohorts of breast cancer patients.

**Contribution** In vitro, DC-SCRIPT suppressed ER- and PR-mediated transcription in a ligand-dependent fashion, whereas it enhanced RARα- and PPARγ-mediated transcription. Breast tumors expressed lower levels of DC-SCRIPT than normal breast tissue from the same patient. Quantification of DC-SCRIPT mRNA expression in three cohorts of breast cancer patients revealed that DC-SCRIPT mRNA expression is an independent prognostic factor for breast cancer patients with ER- and/or PR-positive tumors.

**Implications** DC-SCRIPT is a key regulator of nuclear receptor activity that has prognostic value in breast cancer.

**Limitations** The clinical conclusions about DC-SCRIPT mRNA expression as a prognostic marker in breast cancer were based on nonrandomized retrospective analyses and could not be independently validated in publicly available databases.

*From the editors*

We previously identified and characterized a novel protein, dendritic cell-specific transcript (DC-SCRIPT; also known as ZNF366), that is preferentially expressed by dendritic cells in the immune system.[19,20] DC-SCRIPT contains an amino-terminal proline-rich domain, 11 $Cys_2His_2$-type zinc fingers, and a carboxyl-terminal acidic region. The acidic region of DC-SCRIPT contains a functional binding motif for the corepressor protein CtBP[19,21] and an LXXLL nuclear receptor interaction motif, which is thought to be involved in ER function.[22] Herein, we investigate the effect of DC-SCRIPT on the function of multiple members of the nuclear receptor family as well as the prognostic relevance of DC-SCRIPT expression in breast cancer patients.

## MATERIALS & METHODS

*Cells*

Human embryonic kidney 293 (HEK293) cells were cultured in Dulbecco's modified Eagle (DMEM) medium containing GlutaMAX (Invitrogen, Breda, the Netherlands) supplemented with 10% heat-inactivated fetal calf serum (FCS; Greiner Bio-one, Alphen a/d Rijn, the Netherlands), 1% non-essential amino acids (Invitrogen), and 0.5% antibiotic–antimycotic (Invitrogen). Human hepatocellular carcinoma Hep3b cells were cultured in Iscove's modified Dulbecco's medium (Invitrogen), 10% heat-inactivated FCS, and 0.5% antibiotic–antimycotic. Human breast adenocarcinoma MCF-7 cells were cultured in RPMI-1640 medium (Invitrogen), 10% heat-inactivated FCS, and 0.5% antibiotic–antimycotic. Schneider's Drosophila Line 2 (SL2) cells were grown in Schneider's drosophila medium (Invitrogen) and 10% heat-inactivated FCS. All cell lines were originally obtained from American Type Culture Collection (Manassas, VA). Cells were regularly checked morphologically to verify the identity of each cell line.

*Co-immunoprecipitation Assay*

The nuclear receptors (National Center for Biotechnology Information [NCBI] accession number) ERα (NM_000125), PR-B (NM_000926), RARα (NM_000964), RXRα (NM_002957), and PPARγ (NM_138712) were each cloned into the expression vector pHA-n1 to enable expression of fusion proteins that contain a carboxyl-terminal haemagglutinin (HA) epitope tag. pHA-n1 was generated by replacing the green fluorescent protein (GFP) DNA sequence from the plasmid pEGFP-n3 (Clontech) with the HA DNA sequence. The DNA for DC-SCRIPT (NM_152625) was cloned into the mammalian expression vector pEYFP (Clontech) to enable expression of a DC-SCRIPT fusion protein tagged with enhanced yellow fluorescent protein (YFP). HEK293 cells were plated in 10-cm dishes ($6 \times 10^6$ cells per dish) and incubated for 24 hours. The cells were cotransfected with 5 µg pDC-SCRIPT-EYFP or pEYFP (control) and 5 µg of a vector expressing HA-tagged ERα, PR-B, RARα, or PPARγ by using Metafectene transfection reagent (Biontex, Germany) according to the manufacturer's protocol. The cells were stimulated 8 hours after transfection with the following ligands (all from Sigma, Zwijndrecht, the Netherlands): 10 nM β-estradiol for cells expressing HA-tagged ER; 1 µM all-*trans*-retinoic acid (AtRA) for cell expressing HA-tagged RAR; 1 µM GW1929 (a PPARγ ligand) for cells expressing HA-tagged PPARγ; or the PR ligand 10 nM R5020 (Perkin Elmer, Groningen the Netherlands) for cells expressing HA-tagged PR-B. For all ligands, a 1000-times stock dilution in ethanol was used. Twenty-four hours after transfection, the cells were lysed in radioimmunoprecipitation assay buffer (50 mM NaCl, 1% Triton X-100, 0.1% sodium dodecyl sulfate [SDS], and 50 mM Tris–HCl [pH 7.5]) containing the protease inhibitors 2 µg/mL leupeptin (Sigma, Zwijndrecht, the Netherlands), 2 µg/mL aprotinin (Roche, Woerden, the Netherlands), and 1 mM phenylmethylsulfonyl fluoride (Sigma, Breda, the Netherlands). The cell lysates were used for immunoprecipitation of YFP-tagged proteins with anti-green fluorescent protein (GFP) antibody–coupled protein G beads (GE Healthcare) (this antibody recognizes YFP-tagged proteins). Cells transfected with YFP and HA-tagged ERα, PR-B, RARα, or PPARγ were used as controls for nonspecific binding. Immunoprecipitated proteins were mixed with sample buffer containing 5% glycerol, 6% SDS, 125 mM Tris-HCl [pH 6.8], 0.1 mg/mL bromophenol blue (Gebr. Schmid GmbH + co, Freudenstad, Germany), and 10% β-mercaptoethanol (Sigma, Breda, the Netherlands), heated at 95°C for 5 minutes, and then cooled on ice. The proteins were resolved by electrophoresis on an 8% polyacrylamide gel (ratio of acrylamide to bisacrylamide, 37.5:1) and transferred overnight to Protan nitrocellulose transfer membranes (Schleicher and Schuell, 's Hertogenbosch, the Netherlands) at 30 mA and 4 °C. To block nonspecific protein binding, the membranes were incubated in 2% skimmed milk powder (Campina, Eindhoven, the Netherlands) and 1% bovine serum albumin (BSA) (Roche, Woerden, the Netherlands) in phosphate-buffered saline with 0.01% Tween (PBST) for detecting HA-tagged proteins or in 1% skimmed milk powder and 3%BSA in PBST for detecting YFP-tagged proteins. The membranes were then incubated for 2 hours with a mouse anti-GFP antibody (1:1000 dilution; Roche Applied Science), washed three times in PBST, and subsequently incubated for 1 hour with the secondary antibody IRDye 800CW goat anti-mouse IgG (1:5000 dilution; Li-cor Biosciences) to detect YFP-tagged proteins. To detect HA-tagged proteins, the membranes were incubated with a rat monoclonal anti-HA antibody (1:1000 dilution, clone 3F10; Roche), washed three times in PBST, and incubated for 1 hour with the secondary antibody Alexa Fluor 680–conjugated goat anti-rat IgG (1:5000 dilution; Invitrogen). All membranes were then washed three times in PBST. After staining the YFP- or Ha-tagged proteins, the membranes were scanned by using an Odyssey Infrared Imaging system (Li-cor Biosciences) to visualize the YFP- or Ha-tagged proteins.

*Transcription Assays*

The transcription reporter plasmids pTk-RARE3-luc, which contains three RAR response elements (RAREs) upstream of a firefly luciferase reporter, and pTk-luc were described previously.[23] pAc5.1 (Invitrogen), which contains the Drosophila actin 5C (Ac5) promoter for high-level expression of the gene of interest in SL2 cells,[24] was used to generate the following plasmids for expression in insect cells: pAc-RAR containing RARα, pAc-RXR containing RXRα, and pAc-DCSCRIPT containing DC-SCRIPT. The mammalian expression plasmids pCATCH and pCATCH-DCSCRIPT were described previously[19] and were used in the transcription assays in Hep3b and MCF-7 cells. MMTV-luc, a transcription reporter plasmid containing the mouse mammary tumor virus (MMTV) promoter, which is positively regulated by several classes of steroid hormones including ligands for the PR,[25,26] was kindly provided by Prof. Dr. Stunnenberg (NCMLS, Nijmegen, the Netherlands). MMTV-luc was used to generate MMTV-RLuc, a reporter plasmid in which Renilla luciferase expression is under the control of the MMTV promoter, which was used in the MCF-7 transcription assay. The transcription reporter PPRE-luc (Addgene plasmid number 1015) contains the PPAR response elements upstream of a firefly luciferase reporter and has been described previously.[27] The PR expression plasmid pSG5-PR-B[28] was kindly provided by Prof. Dr. Horwitz (University of Colorado Health Sciences Center) and was used in the transcription assays in Hep3B and MCF-7 cells. The transcription reporter ERE3-TATA-luc (Addgene plasmid number 11354), which contains three copies of the vitellogenin estrogen response element (ERE), has been described previously[29] and was used in the transcription assays in Hep3b cells.

Hep3b cells were plated in 24-well plates ($6 \times 10^4$ cells per well) 8 hours before transfection and all plasmids were transfected into these cells by using a calcium phosphate precipitation kit (Invitrogen) according to the manufacturers protocol. SL2 cells were plated in medium without FCS in 24-well plates ($1 \times 10^6$ cells per well) just before transfection by using a calcium phosphate precipitation kit (Invitrogen). MCF-7 cells were plated in 24-well plates ($5 \times 10^4$ cells per well) 8 hours before transfection and all plasmids were transfected into these cells by the use of Metafectene reagent (Biontex, Germany). Twenty-four hours after transfection, Hep3B, MCF-7, and SL2 cells were stimulated with the RAR ligand AtRA (1 µM) or vehicle (ethanol) for 16 hours. Transfected Hep3B and MCF7 cells were stimulated 16 hours after transfection with the following ligands or vehicle (ethanol) for 24 hours: the ER ligand β-estradiol (10 nM; Sigma, Zwijndrecht, the Netherlands); the PR ligands progesterone (100 nM; Sigma Zwijndrecht, the

Netherlands) or R5020 (10 nM; PerkinElmer, Groningen, the Netherlands); or the PPARγ ligands GW1929 (1 µM; Sigma, Zwijndrecht, the Netherlands) or troglitazone (10 µM; Sigma, Zwijndrecht, the Netherlands). Forty hours after transfection, the cells were lysed in 100 µl Passive Lysis buffer (Promega) and the lysates were analyzed for luminescence with the use of the Dual-Luciferase Reporter Assay System (Promega, Leiden, the Netherlands) according to manufacturer's protocol and a Victor 3 luminometer (PerkinElmer, Groningen, the Netherlands). Relative light units (RLU) were calculated after correction for transfection efficiency based on the activity of a co-transfected reporter plasmid encoding Renilla luciferase under the control of the SV40 promoter (pRL-SV40; Promega). The data are expressed as the mean relative luciferase activity of at least three independent experiments with 95% confidence intervals (CIs).

*Dissociation and Purification of Epithelial Cells from Breast Biopsy Samples*
Normal breast tissue located distally from the tumor was obtained during surgical resection of the primary tumor at the Radboud University Nijmegen Medical Centre (RUNMC) from six anonymous breast tumor patients and was macrodissected within 12 hours of surgical removal and dissociated as previously described;[30] tissue procurement was approved by the institutional ethics committee of the RUNMC. In short, the tissue was minced with a scalpel and dissociated in 0.1% DNase (Roche, Woerden, the Netherlands) and 0.14% collagenase A (Roche) in RPMI-1640 medium, by incubating the minced tissue three times for 45 minutes each time in fresh DNase–collegenase-containing medium. The cell suspension was passed through a 30-µM MACS preseparation filter (Miltenyi Biotec, Utrecht, the Netherlands) washed to remove tissue debris, and stored overnight at 4 °C. The cell suspension was incubated with allophycocyanin (APC)-conjugated anti-human CD326 antibody (Miltenyi Biotec), which recongnizes the epithelial cell surface marker Epithelial Cell Adhesion Molecule (Epcam) followed by incubation with anti-APC MACS beads (Miltenyi Biotect), and the CD326-positive and CD326-negative cells were fractionated by magnetic separation. The CD326-positive and CD326-negative cell fractions were lysed for RNA isolation, and analyzed for the expression of the leukocyte marker CD45, the dendritic cell marker CD11c, the epithelial cell marker CD326, and DC-SCRIPT by means of quantitative polymerase chain reaction (PCR) analysis.

*Complementary DNA (cDNA) Synthesis and Quantitative Polymerase Chain Reaction (PCR) Analysis*
For quantitative PCR analysis of DC-SCRIPT, PBGD, CD45, CD11c, and CD326 mRNA levels, total RNA was isolated from fresh normal breast tissue or cell lines with the use of Trizol reagent (Gibco BRL) according to the manufacturer's protocol. RNA was treated with DNase I (amplification grade, Invitrogen) and reverse-transcribed into cDNA by using random hexamers and Moloney murine leukemia virus reverse transcriptase (Invitrogen). mRNA levels for the genes of interest were determined with a PRISM 7000 sequence detection system (Applied biosystems, Foster City, CA) with SYBR Green (Applied Biosystems) as the fluorophore and the following oligonucleotide primers (forward, reverse): DC-SCRIPT (5'-AAGCATGGAGTCATGGAG-3', 5'-TTCTGAGAGAGGTCAAAGG-3'); PBGD (5'-GGCAATGCGGCTGCAA-3', 5'-GGGTACCCACGCGAATCAC-3'); CD326 (5'-TGTCTGTGAAAACTACAAGCTGG-3', 5'-AGCCATTCATTTCTGCCTTCATC-3'); CD45 (5'-ACCACAAGTTTACTAACGCAAGT-3', 5'-TTTGAGGGGGATTCCAGGTAAT-3'); and CD11c (5'-ATCACCTTCTTGGCTACCT-3', 5'-TGAGGTATTTGGTGAATTGT-3'). We used reaction mixtures and program conditions that were recommended by the manufacturer (Applied Biosystems).

Quantitative PCR data were analyzed with 7000 Systems SDS Software v1.2.3 (Applied Biosystems) and checked for correct amplification and dissociation of the products. mRNA levels of the genes of interest were normalized to mRNA levels of the housekeeping gene porphobilinogen deaminase (*PBGD*),[31] and were calculated according to the cycle threshold method.[32]

*Immunohistochemistry*

Snap-frozen breast cancer specimens from eight anonymous breast cancer patients were obtained from the Rijnstate Hospital (Arnhem, the Netherlands; approved by the institutional ethics committee of the Radboud University Nijmegen Medical Centre) and embedded in OCT embedding matrix (CellPath, Newtown, UK) and sectioned (5-µM thick tissue sections). The sections were placed on Superfrost slides (Thermo Scientific, Ettenleur, the Netherlands), fixed with acetone, and incubated with 4 µg/mL goat anti-human DC-SCRIPT antibody (R&D Systems) followed by incubation with a biotinylated horse anti-goat IgG (Vector Laboratories) and signal development was performed using a Vectastain ABC-AP kit (Vector Labs, Brunswig, Amsterdam) and fast red (Sigma). The epithelial cell surface marker epithelial cell adhesion molecule (Epcam) was detected with a mouse monoclonal anti-CD326 antibody (Miltenyi Biotec) and a biotinylated horse anti-mouse IgG (Vector Laboratories). Isotype-matched goat IgG (R&D Systems) and mouse IgG1 (BD Bioscience) were used as controls. Sections were counterstained with hematoxylin to visualize the cell nuclei and analyzed by using a Leica DM LB microscope (Leica Microsystems B.V., Rijswijk, the Netherlands).

*Patients*

Our use of coded tumor tissues in this study was performed in accordance with the Code of Conduct of the Federation of Medical Scientific Societies in the Netherlands (http://www.federa.org/ [a link to the English version is available at this site]) and was approved by the institutional ethics committee of the Radboud University Nijmegen Medical Centre. All steps in tissue processing and marker assaying were performed by individuals who blinded to the clinical outcomes, and, as much as possible, in accordance with the guidelines for biomarker characterization described by Pepe et al.[33]

From nine anonymous patients who underwent surgery in the early 1990s, both breast tumor and distally located normal breast tissue was macrodissected, and stored in liquid nitrogen until RNA isolation as described below. No other information on these patients or tissues is available.

Other tumor tissues were from patients who had operable unilateral breast cancer and had undergone resection of their primary tumor between November 1987 and December 1997. We included patients who had no previous carcinoma diagnosis, no distant metastases at diagnosis, and no evidence of disease within 1 month after primary surgery. We excluded patients who had received neoadjuvant systemic therapy or who were diagnosed with carcinoma in situ only.

The discovery group (n = 47) was selected from among a previously described cohort of patients[34] based on the availability of tumor RNA and consisted of stage pT1 or pT2[35] ductal (n = 42) or lobular (n = 5) node-negative breast tumors that had received no adjuvant systemic therapy. The tumors were collected, snap frozen, and embedded in OCT compound (Tissue-Tek) as soon as possible after surgical resection (breast conserving lumpectomy: n = 34; modified radical mastectomy: n = 13) at the Rijnstate Hospital (Arnhem, the Netherlands).

Tumors in the validation (n = 97) and tamoxifen-treated (n = 68) groups were also selected based on tumor RNA availability and receipt of adjuvant treatment (no adjuvant systemic treatment [validation group] or adjuvant systemic treatment with tamoxifen) from among a different cohort of patients that was also described earlier [36]. The tumors from this cohort of patients were obtained from a tumor bank in the Department of Chemical Endocrinology (Radboud University Nijmegen Medical Centre) that contains frozen tumor tissue collected from breast cancer patients who were treated at the nine hospitals that form the Comprehensive Cancer Center East in the Netherlands. The tumor tissues were collected by these hospitals for the central measurement of estrogen receptor (ER) and progesterone receptor (PR) levels by ligand binding assay. Patients were defined as ER- and/or PR-positive (level of either or both receptors ≥10 fmol/mg protein) or as ER- and PR-negative (levels of both receptors < 10 fmol/mg protein).

All patients were followed up once every 3 months during the first 2 years after surgery once every 6 months for the next 5 years, and once a year thereafter by means of a medical history, physical examination, and routine laboratory investigations. Each patient received once yearly x-ray mammography, and for those with suspicious results, magnetic resonance imaging of the breast.

*Tumor Tissue RNA isolation*

Aliquots of frozen tissue were pulverized using a microdismembrator (Braun, Melsungen, Germany) and the resulting tissue powder was stored in liquid nitrogen. Total RNA was isolated from 20 mg of tissue powder with the use of an RNeasy Mini kit (Qiagen, Hilden, Germany) with on-column DNase I treatment. Aliquots of RNA were stored at −80 °C. RNA quality was verified by examining the ribosomal RNA bands after agarose gel electrophoresis of each RNA sample and by PCR amplifying PBGD mRNA. RNA concentrations were determined from the spectrophotometric absorption at 260 nm by using a Genequant spectrophotometer (Amersham, Eindhoven, the Netherlands). We observed no effect of storage time in liquid nitrogen on the quality of the RNA.

*Statistical Analyses*

Statistical analyses were carried out using SPSS software (version 16.0; SPSS, Benelux BV). The normality of distributions was tested by the method of Kolmogorov–Smirnov.[37] A normal distribution for DC-SCRIPT mRNA expression values was achieved after taking the $^{10}$log of each value. Differences in the proportions of clinicopathological characteristics among the three patient groups were assessed with Pearson chi-square tests. Differences in PBGD-normalized DC-SCRIPT mRNA expression levels between normal breast tissue and tumor tissue from the same patient were assessed using paired *t* tests, and between tumor tissues and cell lines by unpaired Student *t* tests. Differences in DC-SCRIPT mRNA levels for variables with more than two groups were assessed by analysis of variance. Because data on histological grading were missing for a substantial number of patients, patients with missing data were included in all analyses as a separate group. The disease-free interval (defined as the time from surgery until the diagnosis of recurrent disease) was used as follow-up end point. Contralateral breast cancer or second malignancies were not considered to be recurrent disease. Overall survival was not evaluated because data on breast cancer–specific causes of death were difficult to retrieve from the patient records and, thus, the number of events was too small for reliable statistical analysis. Survival curves were generated by using the Kaplan–Meier method. Cox proportional hazards modeling was used to assess the prognostic value of DC-SCRIPT mRNA expression after dichotomization of the patient group. The proportional hazard assumption was confirmed by examination of the Schoenfeld residuals. An optimal cutpoint was selected in a discovery group at the lowest possible *P* value at such cutpoint, thus representing the best dichotimization of the patient group. The cutpoint was selected by multiple testing and validated in the two other independent patient cohorts. Multivariable Cox proportional hazards modeling was then used to assess the independent prognostic value of DC-SCRIPT mRNA expression in the combined validation and tamoxifen-treated patient cohorts. All clinicopathological variables were entered into the Cox regression model. Subsequently variables that did not contribute to the model ($P > .1$) according to the likelihood ratio statistic were removed in a stepwise fashion until only statistically significant variables remained in the model. Interactions between DC-SCRIPT mRNA expression and ER and PR status and between DC-SCRIPT mRNA expression and tamoxifen treatment were assessed by entering an interaction variable into the model. Two-sided *P* values less than .05 were a priori considered to be statistically significant.

## RESULTS

*Interaction Between DC-SCRIPT and Multiple Nuclear Receptors*

We performed co-immunoprecipitation experiments to examine whether DC-SCRIPT interacts with the steroid nuclear receptors ERα and PR-B or with the RXR heterodimers RARα and PPARγ. For this experiment, whole-cell lysates were prepared from HEK293 cells that had been co-transfected with expression vectors encoding YFP-tagged DC-SCRIPT or YFP (control) and HA-tagged nuclear receptors ERα, PR-B, RARα, or PPARγ. YFP-DC-SCRIPT was immunoprecipitated with anti-GFP antibody–coupled beads, and the immunoprecipitated fraction was subjected to immunoblotting with an anti-GFP antibody. Equal expression of the HA-tagged nuclear receptors was demonstrated by immunoblotting of total lysates of the transfected cells with an anti-HA antibody (figure 1, top panels). YFP-DC-SCRIPT and the control protein YFP were both effectively immunoprecipitated by the anti-GFP antibody–coupled beads (figure 1).

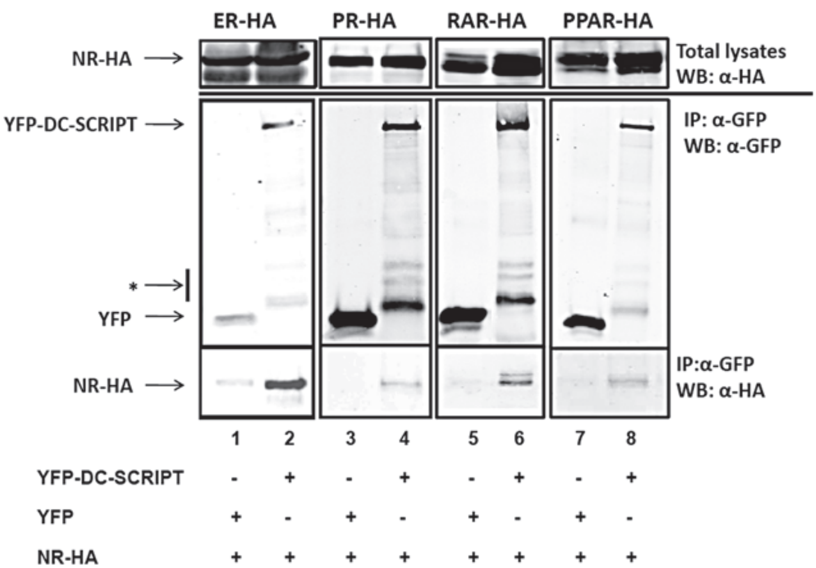
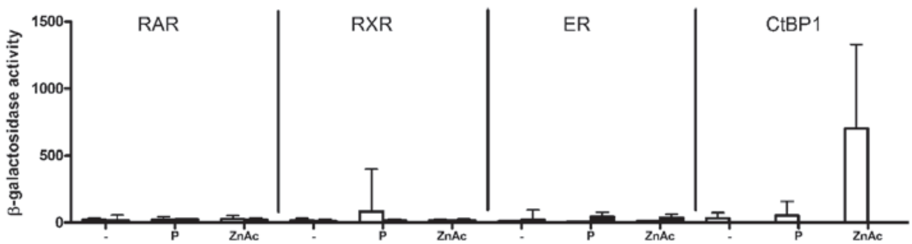



***Figure 1. Association of DC-SCRIPT with multiple nuclear receptors in transfected Hek293 cells**. Lysates from Hek293 cells that were co-transfected with YFP-DC-SCRIPT or YFP and with the indicated HA-tagged nuclear receptors, were immunoprecipitated (IP) with mouse monoclonal anti-GFP antibody–coupled beads (the antibody recognizes YFP-tagged proteins). The immunoprecipitated proteins were subjected to immunoblotting (WB) with the anti-GFP antibody to detect YFP-tagged proteins or with a rat monoclonal anti-HA antibody to detect the co-immunoprecipitated HA-tagged nuclear receptors (NR-HA). Data shown are from one of at least three experiments that produced similar results. YFP-DC-SCRIPT protein degradation products are indicated with an asterisk.*

In addition to the intact YFP-DC-SCRIPT protein, some additional protein bands were also observed that correspond to YFP-DC-SCRIPT breakdown products (figure 1 and data not shown). Interestingly, in cells transfected with YFP-DC-SCRIPT, the HA-tagged nuclear receptor ERα was efficiently co-immunoprecipitated by the anti-GFP antibody–coupled beads (Figure 1, lane 2). HA-tagged PR-B (lane 4), RARα (lane 6), and PPARγ (lane 8) were also specifically co-immunoprecipitated with YFP-DC-SCRIPT, but not with the control protein YFP. We repeated these co-immunoprecipitation experiments using the same stringent lysis conditions in the presence or absence of the ligand and observed no substantial effect on the efficiency of the co-immunoprecipitation experiments (data not shown). Additional yeast two-hybrid experiments showed no direct interaction between DC-SCRIPT and RAR, RXR, or ER (supplementary figure S1). These co-immunoprecipitation data combined with the yeast-two-hybrid results demonstrate that DC-SCRIPT is present in a protein complex with either of these nuclear receptors, and imply that these interactions are indirect and ligand independent.




***Supplementary figure S1. DC-SCRIPT interactions in yeast two-hybrid assays.*** *Yeast two-hybrid assays were performed using bait (- = the negative control empty plasmid , P = proline-rich region of DC-SCRIPT, or ZnAc = the zinc acidic region of DC-SCRIPT) and prey (RAR, RXR, ER, or CtBP1 [positive control for binding to DC-SCRIPT] plasmids in the absence (white bars) and presence (black bars) of ligand (10 µM all-trans-retinoic acid for RAR and RXR prey and 10 nM β-estradiol for ER prey). The physical interaction between the bait and prey proteins activates transcription of a reporter gene that encodes β-galactosidase. β-Galactosidase activity was used as an indicator of protein–protein interactions. β-Galactosidase activity from the yeast clones containing bait and prey was assayed with the use of a Yeast β-Galactosidase Assay Kit (Pierce) and measured using the following equation: (1000 ×* $A_{420}$*)/(t × V ×* $OD_{660}$*), where* $A_{420}$ *= absorbance value at 420 nm, t = time (in minutes) of incubation, V = volume of cells (mL), and* $OD_{660}$ *= optical density at 660 nm. We analyzed 10 colonies per each bait–prey combination per experiment for their β-galactosidase activity, and the data are expressed as the mean values of three experiments; error bars correspond to 95% confidence intervals.*

### *Effect of DC-SCRIPT on ER- and PR-B–Mediated Transcription*

Next, we used luciferase reporter assays to assess the effect of DC-SCRIPT on the transcriptional activity of the steroid-induced nuclear receptors ER and PR. Hep3B cells were transfected with a reporter construct containing response elements for ER (ERE3-TATA-luc)[29] and stimulated with the ER ligand estradiol. In the absence of exogenous ER very little luciferase was produced by transfected cells that were exposed to estradiol (figure 2A, left). By contrast, Hep3B cells that were co-transfected with a mammalian expression vector encoding ER displayed luciferase activity in an estradiol-dependent manner (figure 2A, right). Introduction of increasing amounts of an expression vector encoding DC-SCRIPT into cells expressing exogenous ER revealed a dose-dependent repression of estradiol-dependent ER-mediated luciferase activity (figure 2A, right). These data are in agreement with previous findings that demonstrated that DC-SCRIPT can repress expression levels of endogenous targets of ER (ie, cathepsin D and pS2).[22] Similarly, DC-SCRIPT strongly repressed the hormone-induced (ie, progesterone and R5020) transcription mediated by PR-B on the MMTV promoter in a dose dependent manner (figure 2B). These data indicate that DC-SCRIPT specifically represses transcription mediated by the steroid receptors ER and PR-B in a dose- and hormone ligand–dependent manner.

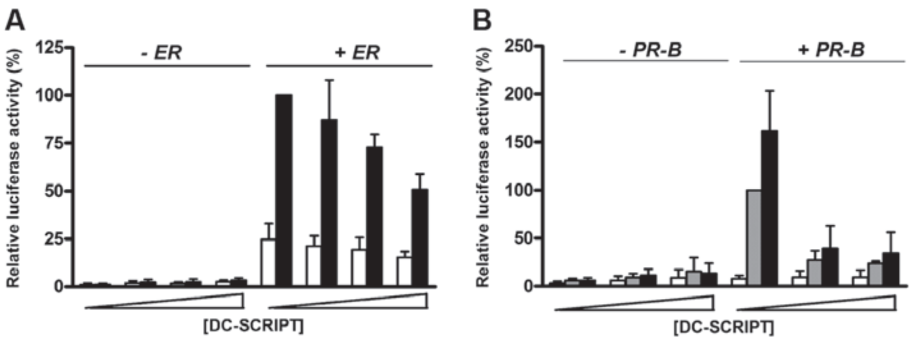



***Figure 2. Effect of DC-SCRIPT on estrogen receptor (ER)– and progesterone receptor (PR)-B–mediated transcription.*** *A) ER-mediated transcription. Hep3b cells were transfected with the firefly luciferase reporter plasmid ERE3-TATA-luc in the absence (−) or presence (+) of a cotransfected ER expression plasmid and increasing amounts of DC-SCRIPT expression plasmid. The cells were treated for 24 hours with vehicle (white bars) or 10 nM β-estradiol (black bars), and luciferase activity was measured with a luminometer. B) PR-B–mediated transcription. Hep3b cells were transfected with the firefly luciferase reporter plasmid MMTV-luc, co-transfected expression vector encoding for PR-B as indicated, and increasing amounts of co-transfected DC-SCRIPT expression vector. The cells were treated for 24 hours with vehicle (white bars), 100 nM progesterone (grey bars), or with 10 nM R5020 (black bars), and luciferase activity was measured with a luminometer. All data were corrected for transfection efficiency by dividing the firefly luciferase values by the value of a co-transfected Renilla luciferase reporter construct. Luciferase data are expressed relative to luciferase production in cells transfected with the nuclear receptor, stimulated with the appropriate ligand, and in the absence of co-transfected DC-SCRIPT. Data are expressed as the mean values of at least four independent experiments; error bars correspond to 95% confidence intervals.*

*Effect of DC-SCRIPT on RARα/RXRα- and PPARγ/RXRα-Mediated Transcription*

To examine the effect of DC-SCRIPT on transcription mediated by two members of the RXR subclass of nuclear receptors, the RXR heterodimers with RARα and PPARγ, we performed additional luciferase reporter assays in Hep3B cells. Hep3B cells transfected with the luciferase reporter construct ptk-RARE3-luc,[23] which contains three response elements for the heterodimer RARα/RXRα, exhibited luciferase activity after stimulation with the RARα/RXRα ligand AtRA (figure 3A). Strikingly, co-expression of increasing amounts of DC-SCRIPT resulted in a dose-dependent increase in luciferase activity upon addition of AtRA (figure 3A), which is in contrast to the repressive effect of DC-SCRIPT on the transcriptional activity of the steroid receptors. To unequivocally demonstrate that the observed increase in luciferase activity was mediated by RARα/RXRα, we repeated the experiment in insect SL2 cells, which do not express RARα or RXRα[38] (figure 3B). DC-SCRIPT activated AtRA-dependent transcription in a dose-dependent fashion in SL2 cells only when RAR/RXR was co-expressed. In addition, removal of the RARα/RXRα response elements in the reporter construct completely abolished luciferase production in both Hep3b and SL2 cells (data not shown). We further demonstrated that DC-SCRIPT enhanced the transcriptional activity of PPARγ/RXRα in Hep3B cells. Hep3B cells transfected with a reporter construct containing the response elements for PPARγ/RXRα (PPRE-luc[27]) failed to display luciferase activity when exposed to the PPARγ ligands GW1929 and troglitazone. However, co-expression of PPARγ/RXRα in these cells resulted in ligand-dependent induction of luciferase activity that was enhanced by DC-SCRIPT in a dose-dependent manner (figure 3C). These data indicate that in contrast to the repressive effect of DC-SCRIPT on the transcriptional activity of the steroid receptors ERand PR-B, DC-SCRIPT enhances transcription mediated by the RXR heterodimers RARα and PPARγ.

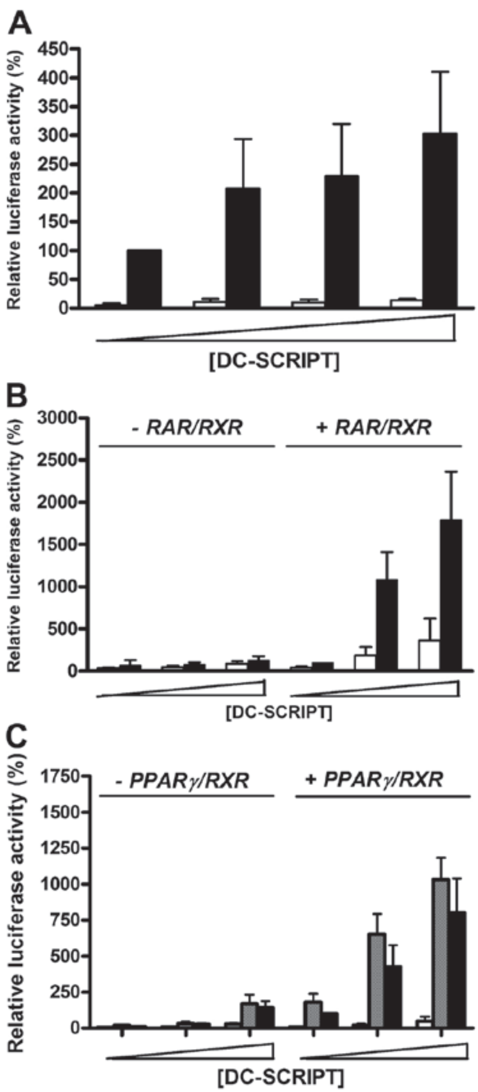
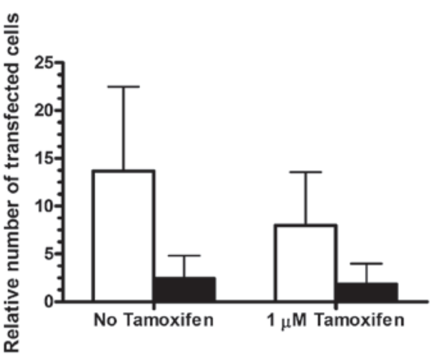



***Figure 3. Effect of DC-SCRIPT on RARα/RXRα- and PPARγ/RXRα-mediated transcription**.*
*A) RARα/RXRα-mediated transcription in Hep3b cells. Hep3b cells were transfected with the firefly luciferase reporter ptk-RARE3-luc and increasing amounts of a co-transfected DC-SCRIPT expression vector. The cells were treated for 16 hours with vehicle (white bars) or 1 µM all-trans-retinoic acid (AtRA) (black bars). Luciferase activity was measured with a luminometer and is expressed relative to luciferase production upon stimulation with AtRA and in the absence of DC-SCRIPT. B) RARα/RXRα-mediated transcription in SL2 cells. SL2 cells were transfected with the reporter firefly reporter ptk-RARE3-luc, the co-transfected expression plasmids RARα and RXRα as indicated and increasing amounts of the expression vector for DC-SCRIPT. The cells were treated for 16 hours with vehicle (white bars) or 1 µM AtRA (black bars). Luciferase activity was measured with a luminometer. C) PPARγ/RXRα-mediated transcription in Hep3b cells. Hep3b cells were transfected with the firefly luciferase reporter PPRE2-luc, the PPARγ expression plasmid as indicated, and increasing amounts of the expression plasmid for DC-SCRIPT. Cell were treated for 24 hours with vehicle (white bars), the PPARγ ligand 1 µM GW1929 (grey bars), or the PPARγ ligand 10µM troglitazone (black bars) Luciferase data (B–C) are expressed relative to luciferase production in the presence of cotransfected nuclear receptor, upon stimulation with the ligand, and in the absence of cotransfected DC-SCRIPT. All data (A–C) are corrected for transfection efficiency by dividing the firefly- luciferase values by the value of a co-transfected Renilla luciferase reporter construct. The data are expressed as the mean of at least four independent experiments; error bars correspond to 95% confidence intervals.*

### *Effect of DC-SCRIPT on nuclear receptor–induced transcription in breast carcinoma MCF7 cells*

Our finding that DC-SCRIPT can repress transcription mediated by the steroid receptors ER and PR-B, whereas on the other hand it can enhance the transcriptional activity of the RXR heterodimers RARα and PPARγ, implies that DC-SCRIPT may be a key regulator that balances the cell's response to multiple nuclear receptor ligands such as vitamins, fatty acids, and hormones. To corroborate these findings, we examined the effect of DC-SCRIPT on nuclear receptor–induced transcription in human breast carcinoma MCF-7 cells which endogenously express both PR and RARα. Therefore, MCF-7 cells were cotransfected with MMTV-Rluc, a progesterone-inducible reporter construct that drives the expression of Renilla luciferase, and ptk-RARE3-luc, an AtRA-inducible reporter construct that drives the expression of firefly luciferase. Control experiments showed that both types of luciferase are induced in cells that are exposed to either the corresponding specific nuclear receptor ligand (PR ligand: R5020 and RARα/RXRα ligand: AtRA) or both nuclear receptor ligands compared with MCF-7 cells treated with vehicle (figure 4A and B). In addition, the effect of DC-SCRIPT on the activity of these endogenously expressed nuclear receptors was in agreement with our findings in transfected Hep3b cells (figure 2B and figure 3A). Interestingly, co-expression of DC-SCRIPT together with both the AtRA-inducible reporter and the progesterone-inducible reporter enhanced RARα/RXRα-mediated transcription (white bars) and concurrently repressed the activity of PR (black bars) (figure 4C). We further found that DC-SCRIPT overexpression inhibited MCF-7 cell growth, as did treatment of MCF-7 cells with the known ER antagonist tamoxifen (supplementary figure S2). These data demonstrate that the presence of DC-SCRIPT can simultaneously modulate the activity of endogenously expressed PR and RARα/RXRα in MCF-7 cells and affects MCF-7 cell growth.




***Supplementary figure S2. Effect of DC-SCRIPT on the growth of MCF-7 cells.*** *Human breast cancer MCF-7 cells were transfected with pEYFP (control, white bars) or pEYFP-DCSCRIPT (black bars) and incubated for 16 hours. The cells were incubated in the presence or absence of 1 μM tamoxifen for 96 hours, harvested and counted using a Bürker cell counting chamber (Optik Labor, Germany) and analyzed for YFP expression by means of fluorescence-activated cell sorting on a FACS-Calibur (BD bioscience) and analyzed using WinMDI 2.8 software by J. Trotter (The Scripps institute). Thereafter, the number of transfected cells was calculated. Number of cells plotted in the graph is relative to the number of cells at the start of incubation in the presence or absence of tamoxifen. Data are expressed as the mean values of three independent experiments; error bars correspond to 95% confidence intervals.*

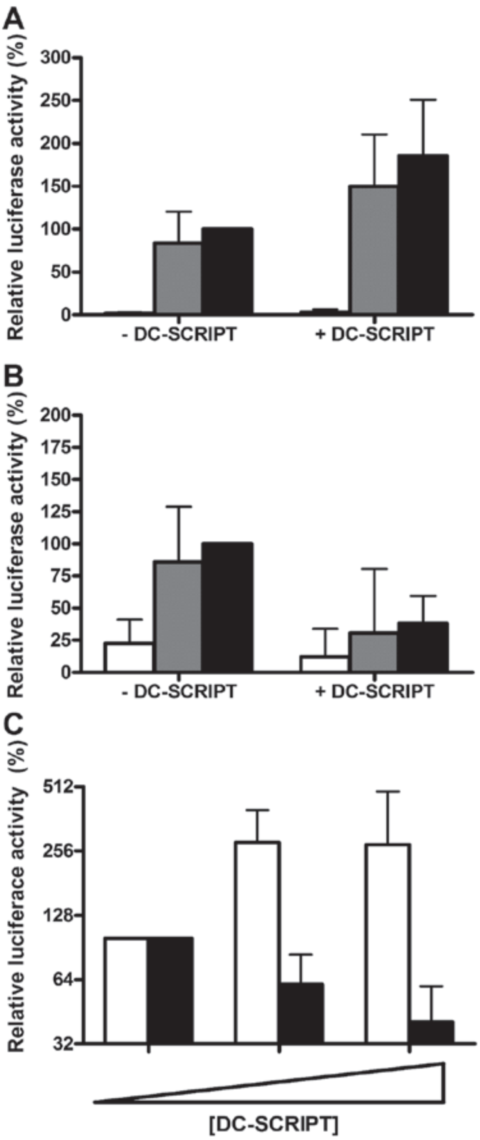



***Figure 4. Effect of DC-SCRIPT on nuclear receptor–induced transcription in breast carcinoma MCF7 cells.***

*A) RARα/RXRα-mediated transcription. MCF7 cells were transfected with the AtRA sensitive reporter ptk-RARE3-luc (firefly luciferase) and in the presence or absence of an expression plasmid of DC-SCRIPT. Cells were treated for 24 hours with vehicle (white bars), with 1 µM AtRA (grey bars), or 1 µM AtRA and 10 nM R5020 simultaneously (black bars). B) PR-B mediated transcription. MCF7 cells were transfected with the progesterone-sensitive Renilla luciferase reporter plasmid MMTV-RLuc and with a DC-SCRIPT expression plasmid as indicated. Cells were treated for 24 hours with vehicle (white bars), with the PR-B ligand 10 nM R5020 (grey bars), or with 1 µM AtRA and 10 nM R5020 simultaneously (black bars). The luciferase data in A and B are expressed relative to luciferase activity in the absence of DC-SCRIPT and upon stimulation with AtRA and R5020. C) RARα/RXRα- and PR-B-mediated transcription. MCF7 cells were co-transfected with the AtRA sensitive ptk-RARE3-luc (firefly luciferase reporter) and with the progesterone-sensitive MMTV-RLuc (renilla luciferase reporter) and with increasing amounts of DC-SCRIPT and stimulated simultaneously for 24 hours with 10nM R5020 and 1 µM AtRA. White bars indicate AtRA-mediated luciferase production (firefly luciferase) and black bars R5020-mediated luciferase production (Renilla luciferase). Luciferase activity is expressed relative to luciferase production upon stimulation with AtRA and R5020 and in the absence of DC-SCRIPT. All luciferase data are expressed as the mean of at least three independent experiments; error bars correspond to 95% confidence intervals*

### *DC-SCRIPT Expression in Breast Epithelial Cells*

Among immune cells, DC-SCRIPT expression appears to be restricted to the dendritic cell lineage.[19] Less is known about DC-SCRIPT expression in non-immune cells. Because DC-SCRIPT regulates transcription mediated by multiple nuclear receptors that play an important role in breast cancer and affects growth of the breast carcinoma MCF-7 cells, we examined DC-SCRIPT mRNA levels in normal breast tissue and corresponding breast tumor tissue from nine patients.

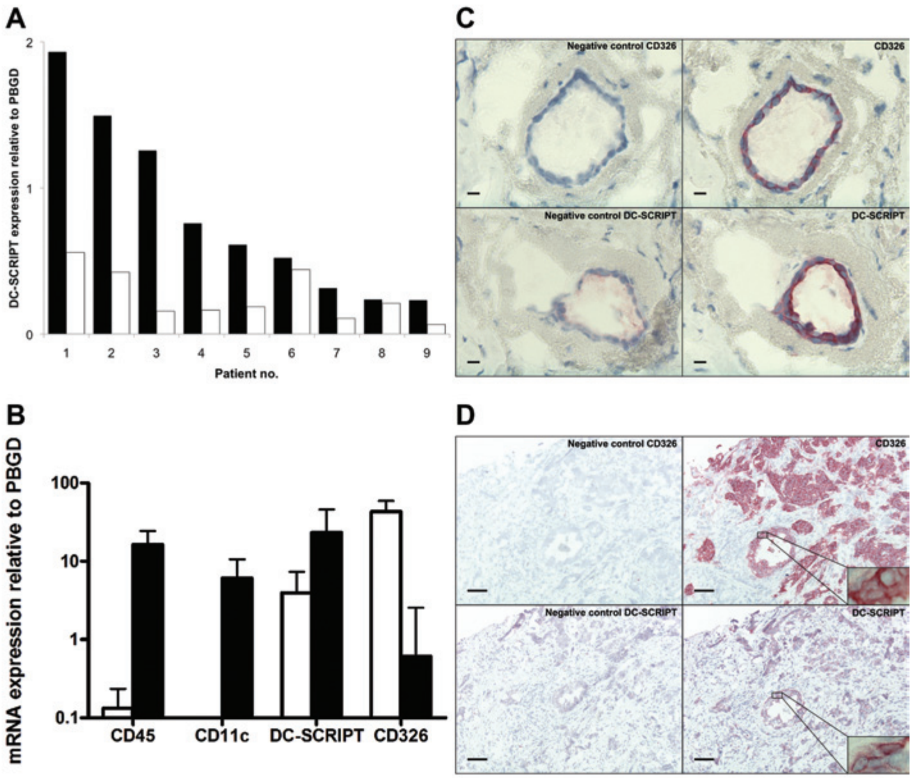



***Figure 5. DC-SCRIPT expression in breast epithelial cells.*** *A) DC-SCRIPT mRNA expression in paired tissue samples. DC-SCRIPT mRNA levels in healthy tissues (black bars) and corresponding breast tumor (white bars) tissue (n = 9 patients) relative to PBGD mRNA as determined by quantitative RT-PCR. B) mRNA expression in CD326-positive and CD326-negative breast cells. Expression of CD326, CD45, CD11c, and DC-SCRIPT mRNA in CD326-positive (white bars) and CD326-negative (black bars) cells purified from breast tissue relative to PBGD mRNA as determined by quantitative real-time RT-PCR. Data from one of the two DC-SCRIPT–positive patients out of six are shown. Data are expressed as the mean of at least three independent quantitative PCR assays; error bars correspond to 95% confidence intervals C,D) DC-SCRIPT and CD326 protein expression in breast tumor sections. Epcam was stained with an anti-CD326 (red), DC-SCRIPT with anti-DC-SCRIPT (red) or matched isotypes (red) as control staining on frozen breast tumor sections as detected by immunohistochemistry staining. Nuclei are counter-stained with hematoxylin (blue). Magnification is indicated by size bars (C) 0.1 µm and D) 1 µm) in lower left corner. Representative sections are shown.*

DC-SCRIPT mRNA expression was readily detected in normal breast tissue by quantitative PCR. Statistically significantly lower DC-SCRIPT mRNA levels were present in the corresponding breast tumor samples (*P* = .010 [paired *t* test]; figure 5A) DC-SCRIPT mRNA was essentially undetectable in more than 50 different cell lines analyzed, including a panel of 16 breast tumor cell lines (data not shown).

To identify which cells in the breast tissue samples expressed DC-SCRIPT mRNA, total cells isolated from fresh normal breast tissue biopsy samples were separated into an epithelial cell–positive and an epithelial cell–depleted fraction by magnetic bead sorting using the epithelial cell marker EpCaM (CD326). Quantitative PCR analysis of these samples showed that, as expected, DC-SCRIPT mRNA was present in the CD326-depleted leukocyte-containing fraction, as were the mRNAs encoding the dendritic cell–specific marker CD11c and the leukocyte marker CD45 (figure 5B). Importantly, DC-SCRIPT mRNA was also detected in the CD326-positive epithelial cell fraction. The absence of CD11c mRNA in the CD326-positive fraction indicates that epithelial cells and not contaminating dendritic cells were the cells that expressed DC-SCRIPT mRNA. Immunohistochemistry on frozen breast tumor sections confirmed that morphologically normal and malignant CD326-positive ductal epithelial cells express DC-SCRIPT protein (figure 5C and D). In concordance with the PCR data (figure 5A), a wide range of DC-SCRIPT protein expression levels was observed in the breast tumor biopsy samples (data not shown).

*Prognostic Significance of DC-SCRIPT mRNA Expression in Breast Cancer*

Next, we explored the prognostic value of DC-SCRIPT mRNA expression in three cohorts of breast cancer patients. Characteristics of these patient cohorts and tumor tissues used in this study are described in table 1. Most patients (70%–80%) in the untreated patient (ie, the discovery and validation) cohorts were postmenopausal at the time of primary surgery, whereas all patients that were treated with tamoxifen were postmenopausal ($P < .001$). The discovery group consisted only of pT1 or pT2 tumors without axillary nodal involvement, whereas both the validation and the tamoxifen cohorts consisted of larger tumors (8%–16% were pT3; $P < .001$) and included patients with positive lymph nodes (validation vs tamoxifen: 17% vs 74%, $P < .001$). The discovery group mostly (72%) underwent breast-conserving lumpectomy, whereas most patients in both the non-treated and tamoxifen-treated cohorts underwent modified radical mastectomy (69%–70%, $P < .001$). Patients treated with endocrine therapy received tamoxifen 40 mg twice daily for at least 2 years. The three study groups did not differ in the relative size of their subgroups as defined by DC-SCRIPT expression (chi-square $P = .543$) (table 1).

First, we measured the DC-SCRIPT mRNA levels in primary breast tumors from a cohort of patients who had not received systemic adjuvant treatment (the discovery group; n= 47).

**Table 1.** Patient and tumor characteristics by study group*

| Characteristic | Discovery N (%) | Validation N (%) | Tamoxifen-treated N (%) | *P*† |
|---|---|---|---|---|
| **Menopausal status** | | | | |
| Premenopausal | 9 (19) | 26 (27) | 0 (0) | <.001 |
| Postmenopausal | 38 (81) | 71 (73) | 68 (100) | |
| **Tumor type** | | | | |
| Ductal | 42 (89) | 61 (63) | 34 (50) | <.001 |
| Lobular | 5 (11) | 10 (10) | 6 (9) | |
| Other/unknown | 0 (0) | 26 (27) | 28 (41) | |
| **Tumor grade‡** | | | | |
| 1 | 7 (15) | 3 (3) | 5 (7) | .026 |
| 2 | 15 (32) | 27 (28) | 20 (29) | |
| 3 | 19 (40) | 33 (34) | 29 (43) | |
| Unknown/missing | 6 (13) | 34 (35) | 14 (21) | |
| **Tumor stage§ (size in mm)** | | | | |
| pT1 (<20) | 31 (66) | 33 (34) | 17 (25) | <.001 |
| pT2 (20–50) | 16 (34) | 55 (57) | 39 (58) | |
| pT3 (>50) | 0 (0) | 8 (8) | 11 (16) | |
| Unknown/missing | 0 (0) | 1 (1) | 1 (1) | |
| **No. of positive lymph nodes** | | | | |
| 0 | 47 (100) | 74 (76) | 5 (7) | <.001 |
| 1–3 | 0 (0) | 7 (7) | 38 (56) | |
| >4 | 0 (0) | 10 (10) | 12 (18) | |
| Unknown/missing | 0 (0) | 6 (6) | 13 (19) | |
| **ER and PR status** | | | | |
| ER and PR negative | 10 (21) | 32 (33) | 21 (31) | .342 |
| ER and/or PR positive | 37 (79) | 65 (68) | 47 (69) | |
| **Type of surgery** | | | | |
| Lumpectomy | 34 (72) | 29 (30) | 21 (31) | <.001 |
| Mastectomy | 13 (28) | 68 (70) | 47 (69) | |
| **Radiotherapy** | | | | |
| No | 14 (30) | 35 (36) | 7 (10) | .001 |
| Yes | 33 (70) | 62 (64) | 61 (90) | |
| **Systemic adjuvant therapy** | | | | |
| No | 47 (100) | 97 (100) | 0 (0) | <.001 |
| Yes | 0 (0) | 0 (0) | 68 (100) | |
| **DC-SCRIPT mRNA level‖** | | | | |
| Low | 16 (34) | 33 (34) | 18 (26) | .543 |
| High | 31 (66) | 64 (66) | 50 (74) | |

*ER = estrogen receptor; PR = progesterone receptor.

† Chi-square test (two-sided).

‡ Bloom–Richardson grade.[39]

§ American Joint Committee on Cancer staging.[35]

‖ DC-SCRIPT/PBGD ratio higher or lower than 0.15, the optimal prognostic cutpoint in the discovery group.

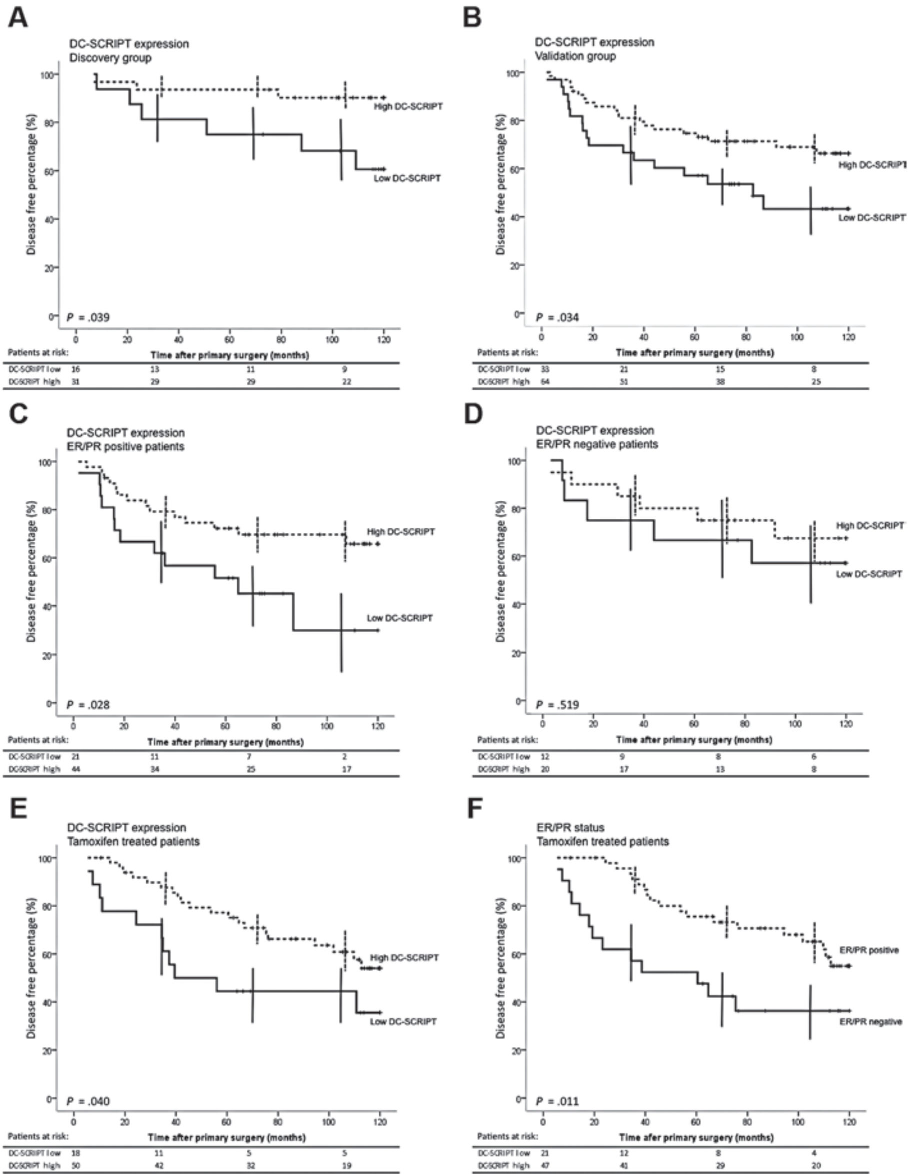


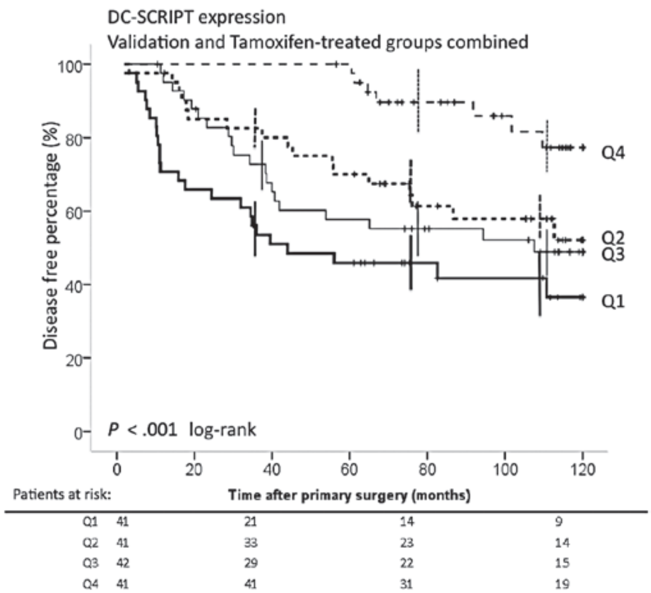

***Figure 6. DC-SCRIPT expression as a prognostic marker in ER- and PR-positive breast cancer.*** *A) Kaplan–Meier analysis of disease-free interval according to DC-SCRIPT mRNA expression in tumors from breast cancer patients who did not have axillary lymph node metastases and did not receive systemic adjuvant therapy (discovery group; n = 47). B) Kaplan–Meier analysis of disease-free interval according to DC-SCRIPT mRNA expression in tumors from an independent validation group that included patients with larger tumors than those in the discovery group and with axillary lymph node metastases (n = 97). C) Kaplan–Meier analysis of disease-free interval according to DC-SCRIPT mRNA expression in tumors from patients in the validation group with ER- and PR-positive tumors (n = 65). D) Kaplan–Meier analysis of disease-free interval according to DC-SCRIPT mRNA expression in tumors from patients in the validation group with*

*ER- and PR-negative tumors (n = 32). E) Kaplan–Meier analysis of disease-free interval according to DC-SCRIPT mRNA expression in tumors from patients treated with tamoxifen (n = 68). (A–E) Patients with high DC-SCRIPT expression were compared with patients with lower DC-SCRIPT expression. High DC-SCRIPT expression (dotted line) indicates expression above the optimal cutoff of DC-SCRIPT/PBGD transcript ratio of 0.15; low DC-SCRIPT expression (solid line) indicates expression below DC-SCRIPT/PBGD transcript ratio of 0.15. F) Kaplan–Meier analysis of disease-free interval according to tumor ER and PR status among patients treated with tamoxifen (n = 68). Dotted line corresponds to patients with ER- and/or PR-positive tumors; solid line corresponds to patients with ER- and PR-negative tumors. All P values are two-sided (Cox proportional hazards tests). Tick marks indicate censored events, and vertical lines indicate 95% confidence intervals. Numbers below graphs are the number of patients at risk at that time point.*

The DC-SCRIPT mRNA level in this patient group was not associated with any of the clinicopathological parameters [ie, menopausal status, tumor type, grade,[39] tumor size, lymph node status, hormone receptor status, or type of surgery or therapy or with the percentage of tumor cells in the biopsy sample (data not shown). We then analyzed the prognostic value of DC-SCRIPT mRNA level in the discovery group after dichotomization of the patients according to an optimal DC-SCRIPT to PBGD transcript ratio cutoff of 0.15, which resulted in 16 (34%) patients with low levels of DC-SCRIPT mRNA and 31 (66%) patients with high levels of DC-SCRIPT mRNA in their primary tumors. In a Kaplan–Meier survival analysis, these two groups of patients differed statistically significantly with respect to disease-free interval: those with a high DC-SCRIPT mRNA level had a statistically significantly longer disease-free interval than those with a low DC-SCRIPT mRNA level (hazard ratio [HR] of recurrence = 0.23, 95% confidence interval [CI] = 0.06 to 0.93, $P$ = .039) (figure 6A).

To validate this finding, we analyzed DC-SCRIPT expression in an independent Cohort of patients who also had not received adjuvant systemic treatment (the validation group; n = 97). Overall, the validation group included more patients with advanced disease than the discovery group (eg, pT3 tumors: 8% vs 0%; axillary nodal involvement: 17% vs 0%) (table 1). Nevertheless, when we applied the same cutoff, the 64 patients (66%) with a high tumor DC-SCRIPT mRNA level had statistically significantly better prognosis than the 33 patients (34%) with low tumor DC-SCRIPT mRNA level (HR of recurrence = 0.50, 95% CI = 0.26 to 0.95, $P$ = .034) (figure 6B).

Because DC-SCRIPT represses the activity of both ER and PR, we explored the prognostic value of DC-SCRIPT in relation to the ER and PR status of the primary tumor. We found that the tumor DC-SCRIPT mRNA level had statistically significant prognostic value for patients in the discovery and validation groups with ER- and/or PR-positive tumors but not for those with ER- and PR-negative tumors (discovery group, patients with ER- and/or PR-positive tumors: HR of recurrence = 0.16, 95% CI = 0.03 to 0.89, $P$ = .030; discovery group, patients with ER- and PR-negative tumors: HR of recurrence = 0.73, 95% CI = 0.07 to 8.07, $P$ = .797; validation group, patients with ER- and/or PR-positive tumors: HR of recurrence = 0.42, 95% CI = 0.19 to 0.91, *P* = .028 [figure 6C]; validation group, patients with ER- and PR-negative tumors: HR of recurrence = 0.68, 95% CI = 0.21 to 2.22, *P* = .519 [figure 6D]).




***Supplementary Figure S3. DC-SCRIPT expression as a prognostic marker in breast cancer.***
*Kaplan–Meier estimates of disease-free interval according to quartiles of DC-SCRIPT mRNA expression in tumors from patients in the combined validation and tamoxifen-treated groups (n = 165). Q1 is the lowest quartile of DC-SCRIPT mRNA expression, and Q4 is the highest quartile.*

We next assessed the prognostic value of DC-SCRIPT expression and tumor ER and PR status in a third cohort of patients who received anti-estrogen therapy with tamoxifen (n = 68). Strikingly, when we applied the same cutoff that was established in the discovery group, DC-SCRIPT expression had approximately the same prognostic value in the tamoxifen-treated cohort as was found in the validation group (HR of recurrence = 0.46, 95% CI = 0.22 to 0.97, *P* = .040) (figure 6E). As expected, tumor ER and PR status predicted the response to endocrine therapy in this group of patients (*P* = .011, figure 6F).

We next performed univariate and multivariable Cox regression analyses in the combined validation and tamoxifen cohorts (n = 165) to assess the independence of the prognostic value of DC-SCRIPT mRNA expression after correction for standard clinicopathological parameters (table 2). In the univariate analysis, tumor grade (*P* = .022), tumor size (*P* = .020), lymph node status (*P* < .001), the type of surgery (*P* =.018), and DC-SCRIPT mRNA level (*P* = .004) were statistically significantly associated with prognosis but tumor ER and PR status was not. In this combined cohort, DC-SCRIPT mRNA level was statistically significantly associated with prognosis when entered as a continuous variable in a Cox regression analysis independent of cutpoint (*P* = .013;

data not shown) and after dividing the patients into quartiles of DC-SCRIPT mRNA level (*P* = .001, log-rank test for trend; supplementary figure S3, available online). The multivariable analysis (table 2) revealed that DC-SCRIPT is an independent factor contributing to prognosis after correction for tumor size, lymph node status, systemic adjuvant treatment (n = 145; HR of recurrence = 0.50, 95% CI = 0.29 to 0.85, *P* = .010).

The interaction between DC-SCRIPT mRNA level and tamoxifen treatment was not statistically significant (*P* = .470), whereas the interaction between DC-SCRIPT mRNA level and tumor ER and PR status was (*P* = .017) (table 2). These data indicate that the prognostic value of DC-SCRIPT mRNA level is independent of whether or not the patient received tamoxifen, but does depend on whether or not the tumor expresses ER and PR.

**Table 2.** Univariate and multivariable Cox proportional hazards modeling of factors associated with disease-free survival in the combined validation and tamoxifen-treated patient groups (n=165)*

| Factor and comparison | Univariate analysis | | Multivariable analysis | |
|---|---|---|---|---|
| | **HR of recurrence (95% CI)** | ***P*†** | **HR of recurrence (95% CI)** | ***P*†** |
| Postmenopausal vs premenopausal | 0.69 (0.38 to 1.26) | .224 | 0.58 (0.27 to 1.24) | .157 |
| Tumor type | | | | |
| Lobular vs ductal | 1.94 (0.96 to 3.92) | .216 | 1.18 (0.45 to 3.10) | .771 |
| Other or unknown vs ductal | 1.03 (0.61 to 1.75) | | 1.25 (0.67 to 2.32) | |
| Tumor grade ‡ | | | | |
| 2 vs 1 | 0.94 (0.21 to 4.19) | .022 | 0.86 (0.18 to 4.03) | .337 |
| 3 vs 1 | 2.29 (0.55 to 9.57) | | 1.67 (0.38 to 7.33) | |
| Tumor stage § | | | | |
| pT2 vs pT1 | 1.73 (0.96 to 3.13) | .020 | 1.74 (0.91 to 3.32) | .133 |
| pT3 vs pT1 | 2.97 (1.39 to 6.37) | | 2.19 (0.96 to 5.02) | |
| No. of positive lymph nodes | | | | |
| 1–3 vs 0 | 1.63 (0.89 to 2.97) | <.001 | 4.13 (1.86 to 9.16) | <.001 |
| >4 vs 0 | 4.68 (2.48 to 8.84) | | 11.6 (5.17 to 26.2) | |
| ER- and/or PR-positive vs ER- and PR- negative | 0.81 (0.49 to 1.33) | .411 | 0.95 (0.52 to 1.75) | .879 |
| Mastectomy vs lumpectomy | 1.93 (1.09 to 3.43) | .018 | 0.71 (0.29 to 1.70) | .439 |
| Radiotherapy (yes vs no) | 1.23 (0.69 to 2.17) | .480 | 0.59 (0.29 to 1.21) | .149 |
| Systemic adjuvant therapy (yes vs no) | 1.14 (0.71 to 1.84) | .593 | 0.33 (0.16 to 0.67) | .002 |
| DC-SCRIPT mRNA level (high vs low)‖ | 0.49 (0.30 to 0.80) | .004 | 0.50 (0.29 to 0.85) | .010 |
| DC-SCRIPT interaction | | | | |
| With systemic adjuvant therapy | 0.83 (0.49 to 1.39) | .470 | NA | |
| With ER and PR status | 0.56 (0.34 to 0.91) | .017 | NA | |

* HR = Hazard ratio; ER = estrogen receptor; PR = progesterone receptor; NA = not applicable.
† Cox proportional hazards
‡ Bloom–Richardson Grade[39].
§ American Joint Committee on Cancer staging[35]
‖ DC-SCRIPT/PBGD ratio higher or lower than 0.15, the optimal prognostic cutpoint in the discovery group.

## DISCUSSION

Here we have identified DC-SCRIPT as a regulator of the activity of several subclasses of nuclear receptors and as a prognostic marker for ER- and/or PR-positive breast cancer. We have shown that expression of DC-SCRIPT in Hep3B cells and MCF-7 cells represses the hormone-induced activity of ER and PR while it concurrently enhances RARα/RXRα-and PPARγ/RXR-mediated transcription. In addition, we showed that ductal epithelial cells express DC-SCRIPT mRNA, that breast tumors express lower levels of DC-SCRIPT than normal breast tissue from the same patient, and that breast tumor cell lines do not express DC-SCRIPT mRNA. Moreover, quantification of DC-SCRIPT mRNA expression in three cohorts of breast cancer patients revealed that DC-SCRIPT mRNA expression is an independent prognostic factor for breast cancer patients with ER- and/or PR-positive tumors.

To our knowledge, we have provided the first evidence for the presence of DC-SCRIPT in multiple nuclear receptor protein complexes based on data from co-immunoprecipitation experiments. Results of yeast two-hybrid experiments imply that DC-SCRIPT, unlike its direct binding to CtBP1,[19] does not bind directly to nuclear receptors. This finding suggests that the interaction between DC-SCRIPT and nuclear receptors is likely to be mediated by other nuclear receptor co-regulators that are present in these large multi-protein complexes.[14] Because DC-SCRIPT was previously shown to interact with multiple proteins known to be present in these protein complexes,[19,22] it will be interesting to investigate the molecular mechanism by which DC-SCRIPT differentially modulates the activity of multiple classes of nuclear receptors.

Because malfunction of nuclear receptors and their co-regulators has been associated with breast cancer,[2-4] we evaluated the expression of DC-SCRIPT in breast tissue. We found that DC-SCRIPT is expressed by normal breast tissue whereas less DC-SCRIPT mRNA could be detected in the corresponding breast tumor tissue. Combined with our finding that DC-SCRIPT inhibits cell growth of the breast carcinoma cell line MCF-7, these data suggest that DC-SCRIPT may act as a tumor suppressor in breast cancer development. Such a function for DC-SCRIPT is also consistent with our finding that DC-SCRIPT overexpression in Hep3B and MCF-7 cells inhibits the activity of ER and PR, which were previously shown[6] to exhibit proliferative and anti-apoptotic activities in breast cancer cells. In contrast, RARα/RXRα and PPARγ/RXR, the transcriptional activities of which are enhanced by DC-SCRIPT, are reported to have anti-proliferative and pro-apoptotic effects in breast cancer cells.[40-42] Moreover, a recent report showed that expression of RARα/RXRα target genes identified in MCF-7 cells predicted a positive clinical outcome in breast cancer patients.[43] Our findings in MCF-7 cells indicate that DC-SCRIPT affects the balance in the activities of endogenous PR and RARα/RXRα in favor of RARα/RXRα activity when the breast cancer cells are simultaneously stimulated with the respective ligands. To directly demonstrate a tumor suppressor function for DC-SCRIPT and to assess the impact of DC-SCRIPT expression on transcription at a genome-wide level is very difficult because prolonged overexpression of DC-SCRIPT in all of the cell lines tested thus far resulted in growth inhibition and death of the DC-SCRIPT-expressing cells (data not shown). Moreover, none of the cell lines analyzed endogenously expressed DC-SCRIPT, which prevented us from performing the obvious knock-down experiments of endogenous DC-SCRIPT to further investigate its anti-proliferative effect. It will therefore be important to define the conditions and factors that regulate DC-SCRIPT expression, from both a physiological and a therapeutic perspective.

To our knowledge, this is the first time that DC-SCRIPT mRNA expression has been identified as a prognostic marker in breast cancer. Over the years, molecular profiling has yielded genetic signatures for many solid tumors[44-49] including breast cancer,[50] however DC-SCRIPT was not present in these signatures. The absence of DC-SCRIPT from these signatures can be explained by the fact that on older microarrays used to determine breast cancer signatures, the DC-SCRIPT gene was not yet present and the relatively low DC-SCRIPT mRNA expression may have prevented its detection in more recent studies. Our discovery of DC-SCRIPT as an independent prognostic marker for breast cancer patients with ER- and/or PR-positive tumors was possible because we used a unique cohort of patients who were treated with systemic adjuvant therapy and followed up for 10 years.[34,36] Prognostic significance can only be ascertained in such patient groups,[51,52] which are becoming increasingly scarce because currently even patients with negative axillary lymph nodes almost all receive systemic adjuvant therapy. Our finding that DC-SCRIPT has prognostic value independent of a specific treatment suggests that DC-SCRIPT may contribute to tumor growth characteristics and is consistent with the proposed tumor suppressor function of DC-SCRIPT. The potential clinical significance of DC-SCRIPT extends beyond untreated patients given that we showed that high DC-SCRIPT expression, like ER and PR status, is also prognostic in tamoxifen-treated patients. In line with this finding is our demonstration that DC-SCRIPT inhibited the growth MCF-7 cells treated without tamoxifen or with tamoxifen to block ER function. These data imply that DC-SCRIPT expression may be used to select ER- and/or PR-positive patients who might be candidates for more aggressive adjuvant therapy.

This study has several limitations. First, we have not found independent validation of DC-SCRIPT mRNA expression as a prognostic marker in breast cancer in publicly available databases; the absence of the DC-SCRIPT gene on older microarrays and the relatively low abundance of DC-SCRIPT mRNA may have prevented its detection. Second, our clinical conclusions are therefore based on nonrandomized retrospective analyses. To attain a higher level of evidence, independent multicenter and/or randomized prospective studies of DC-SCRIPT expression analyses are necessary.

It will be extremely interesting to assess in future validating studies whether the RARα and/or PPARγ status of the tumors is of relevance regarding the prognostic value of DC-SCRIPT. The presence of RARα/RXRα target genes in the genetic signature of breast tumor samples predicts a positive clinical outcome in breast cancer patients.[43] Stimulation of the nuclear receptors RAR/RXR and PPARγ/RXR has been explored as a novel therapy for breast cancer.[53,54] So far these therapies have shown only limited success due to retinoic acid resistance.[18,55] Intriguingly, our data demonstrate that DC-SCRIPT is able to simultaneously enhance the activities of RARα/RXRα and PPARγ/RXRα and repress the activities of ERα and PR-B. On the basis of these findings we hypothesize that the anti-proliferative effect of DC-SCRIPT in breast cancer cells is mediated by modulating the activity of multiple nuclear receptors. It will be interesting therefore to also examine DC-SCRIPT expression levels in clinical trials that have explored the effect of stimulation of the RAR/RXR and PPARγ/RXR on the clinical outcome in breast cancer patients.

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# Chapter 6

## Crosstalk and DC-SCRIPT: expanding nuclear receptor modulation

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## ABSTRACT

Nuclear Receptors (NR) are intracellular receptors that execute a transcriptional program upon binding to hormones, vitamins and metabolic products. They are key regulators of distinct physiological processes, including growth and differentiation, metabolism, and immunity. The impact of NR activation on a given cell can differ from proliferation induction to programmed cell death. NR malfunction is associated with different diseases, such as diabetes, chronic inflammatory diseases and cancer. Much progress has been made towards understanding the transcriptional regulation by individual NR at the molecular level. However, essentially every cell expresses multiple NR and will encounter complex mixtures of NR ligands during its life cycle. In this review, we will focus on novel insights in balancing NR activity via NR crosstalk and DC-SCRIPT/ZNF366, a bi-functional NR coregulator. The impact on breast cancer development and prognosis will be discussed.

## INTRODUCTION

In a physiological context cells are not alone, they communicate with surrounding cells and interact with the extracellular matrix (ECM). Within their local environment they experience complex mixtures of compounds and substrates, including growth factors, hormones, vitamins, organic constituents and their metabolic products. These cellular cues and the nature of the ECM offer a highly dynamic setting for cells. To maintain a healthy phenotype and avert malignant cell growth, cells require strict regulation of cell growth, -division and -death, processes in which nuclear receptors (NR) play an important role. In fact, dysfunction of NR can lead to uncontrolled proliferation and cell survival, allowing for cancer development.

The NR family of ligand-inducible transcription factors recognize a diversity of ligands, including hormones (*e.g.* estrogens, corticosteroids and androgens), vitamins (*e.g.* vitamin A and D) and metabolic products (*e.g.* fatty acids, eicosanoid derivatives and oxidized lipids).[1] In men 48 family members have been described that all share common functional domains: they contain an amino-terminal transactivation domain, a DNA binding domain (DBD) and a carboxy-terminal ligand-binding domain.[2-4] Based on their mode of action NR are divided into two main subclasses. Type I NR comprise the steroid receptors, including the Estrogen Receptor (ER), Progesterone Receptor (PR), Androgen Receptor (AR) and Glucocorticoid Receptor (GR). Steroid receptors are classically sequestered in the cytoplasm of cells by binding to heat shock proteins. Upon ligand binding, they homodimerize and translocate to the nucleus where they bind to specific DNA sequences. Binding to these DNA response elements will lead to recruitment of coactivator complexes ultimately resulting in transcriptional activation of the target gene. Type II NR consists of the Retinoid X Receptor (RXR) heterodimers. Well-known members of this class are the Retinoic Acid Receptor (RAR), peroxisome proliferator-activated receptors (PPAR) and the Vitamin D Receptor (VDR). In contrast to type I NR, type II receptors generally reside in the nucleus in the absence of ligand. They bind to their response elements and are often complexed with corepressor proteins. Upon ligand binding, conformational changes occur, corepressor proteins are released, coactivators are recruited and transcription is initiated. (For reviews, see [2-6])

Pathways affected by both type I and type II NR signalling in cells include those involved in apoptosis, cell cycle regulation and growth factor signalling. This has been particularly well-studied in the development and progression of breast cancer,[7] where the type I NR ER and the type II NR RAR exert opposing effects on these pathways. Typically, estrogens are referred to as pro-tumourigenic, displaying proliferative and anti-apoptotic effects via ER activation, whereas RAR stimulation by retinoids is considered anti-tumourigenic, repressing cell growth while inducing differentiation and/or apoptosis. These observations indicate that balancing the activity of NR is of major importance in keeping a healthy cell phenotype. The effects of a particular NR ligand on a cell will thus not only depend on its own concentration, the expression levels of its receptor and its coregulators, but also on the presence or absence of other ligands, other NR and their coregulators. Indeed, NR coregulators, like the coactivators SRC-1 (steroid receptor coactivator 1) and AIB1 (amplified in breast cancer-1 also known as SRC3) and NR corepressors (NcoRs) play a key role in regulating the cell's response to NR ligands and have been associated with breast cancer.[7,8] They play a central role in NR crosstalk, customizing the effect of NR to each target in relation to the local environment.

A protein termed dendritic cell-specific transcript (DC-SCRIPT or ZNF366) was first described in 2006 and recently shown to act as a coregulator of multiple NR.[9] DC-SCRIPT is a highly conserved protein[10,11] originally identified in a unique immune cell subset, the antigen presenting dendritic cells (DC).[12] The DC-SCRIPT protein consists of an N-terminal proline-rich region, 11 $Cys_2His_2$-type zinc fingers and a C-terminal acidic region. Beyond its zinc fingers, it shares no homology with proteins alike and has a restricted expression pattern, including epithelial cells. Remarkably, DC-SCRIPT could regulate the activity of several subclasses of NR. DC-SCRIPT overexpression inhibited the activity of ER and PR, known for their proliferative and anti-apoptotic activities in breast cancer cells. In contrast, the transcriptional activities of RAR/RXR and PPAR/RXR, mostly known for their anti-proliferative and pro-apoptotic effects in breast cancer cells, were enhanced by DC-SCRIPT. In this review, we will focus on NR crosstalk and DC-SCRIPT. The importance of balancing NR activity in breast cancer development and prognosis will be discussed.

## NUCLEAR RECEPTOR COREGULATORS

Balancing NR activity is of major importance in keeping a healthy cell phenotype. It is becoming more and more apparent that the composition of NR coregulator complexes is crucial for maintaining this balance, as these complexes are essential for appropriate NR responses to ligands and other extra- and intracellular signals. Coregulator complexes are involved in multiple transcriptional processes such as catalyzing the process of chromatin condensation and facilitating the communication with the general transcription apparatus at target gene promoters.[13] Coregulators are classically divided into coactivators known to enhance NR-mediated transcription and corepressors that dampen the agonistic effect of NR ligands. Coactivator proteins can be present in three different multiprotein complexes, i) in the SWI/SNF complex that is associated with ATP-dependent alteration in chromatin structure, ii) in the p160/CBP complex which is mostly related to histone acetylation and iii) in the mediator complex which is involved in the activation of RNA polymerase II and the initiation of transcription. Conversely, corepressors can inhibit NR-mediated transcription by interfering with the access of coactivators or by associating with HDAC (histone deacetylase) complexes that repress transcription by catalyzing the condensation of chromatin. Initially it was thought that corepressors and coactivators reside in distinct protein complexes. However, current studies imply that these molecules can exist in the same large complexes suggesting that transcription repression and activation are more closely integrated than initially suggested.[14,15] Depending upon cell and signalling context, coactivators and corepressors can on occasion switch roles.[16-19] Evidence indicates that the operational definition between repressors and activators can be modified by gene, cell, and signalling context for any one coregulator. Posttranslational modification is an important mode of regulation. Phosphorylation, acetylation, ubiquitination, methylation and/or sumoylation of NR but also of their coregulators have been reported to regulate the assembly, dissociation and the content of the regulatory protein complexes. These modifications allow for the dynamic modulation and integration of extracellular signalling pathways that will ultimately enhance or decrease the transcriptional efficacy of NR-cofactor containing complexes.[13,20] The complex network of coactivators and corepressors thus provides a balanced and sensitive control mechanism to regulate NR target gene expression.

A major boost in understanding NR function has been obtained by the introduction of genome wide ChIP (Chromatin ImmunoPrecipitation) technology. This technique enables the identification of specific transcription factors and the kinetics of cofactor recruitment to a specific locus. The order of recruitment, information on the local chromatin structure and the epigenetic state can also be determined. Especially the ERα-mediated transcriptional regulation has been elucidated by the use of multiple ChIP profiling studies. Excellent reviews detailing the recent advances in the identification of the ERα-binding sites, their target gene network and clinical applications can be found elsewhere.[21,22] A perfect example for the identification of new factors involved in ER transcriptional regulation using these new techniques is the FoxA1 protein. FoxA1 is involved in ERα mediated transcriptional regulation by acting as a pioneer factor. Through its chromatin-remodelling activity, FoxA1 allows for the opening of genomic regions in the absence of hormone.[23-26] FoxA1 binds DNA adjacent to response elements for ER, thereby facilitating the local recruitment of ER.[27] FOXA1/ER binding sites are often located in distal enhancers far from the transcription start site of target genes. Gene transcription thus requires chromatin looping to allow physical contact between the protein complexes binding to the enhancer and the proximal promoter.[27,28] Recently, FoxA1 binding was suggested to be required but not sufficient to trigger functional activity of a given (cis)-regulatory region.[29] The cellular and physiological context co-determines whether FoxA1-bound enhancers are active or inactive.[29] The number of ER binding sites in MCF-7 cells identified by ChIP that do contain a FOXA1 motif has variably been estimated from less than 10% to approximately halve of the ER binding sites.[23-26,28,30,31] The important role of FoxA1 in NR regulation is further emphasized by the finding that also AR activity is regulated by this pioneer factor.[32,33]

## FUNCTIONAL IMPACT OF NUCLEAR RECEPTOR ACTIVATION

NR activation induces a plethora of different proteins that take part in distinct biological pathways. Dysfunction of NR and/or their coregulators may affect one or more of these pathways and can lead to uncontrolled proliferation and cell survival, allowing for cancer development. Dominant pathways affected by NR in oncogenic cells include the apoptosis pathway, cell cycle regulation and growth factor signalling. These pathways have been particularly well studied in the development and progression of breast cancer, where the type I NR ER and the type II NR RAR exert opposing effects.

Estrogens proliferative effects are held to be responsible for its role as a causative agent in breast cancer.[34] In the breast cancer cell line MCF-7, ERα induces the $G_1$- to S-phase transition, accelerating cell proliferation.[35] This is driven by the increased activity of the ERα responsive cell cycle-related genes c-myc, cyclin D1 (CCND1), cyclin E1 (CCNE1) and E2 (CCNE2).[25,34,36] siRNA mediated knock-down of either CCND1, CCNE1 or CCNE2 abrogates estrogen induced proliferation in breast cancer cells.[34] Repressive cell cycle-regulators such as cyclin G2 (CCNG2) are also affected by ERα activation. Binding of ERα to the CCNG2 promoter initiates corepressor recruitment, negatively regulating expression of CCNG2.[37] A potent feed forward loop has been reported for the autocrine protein trefoil factor-1 (TFF1), a direct ERα target. TFFs are overexpressed in several solid tumours including breast. They function in wound healing, where they promote migration and prevent apoptosis.[38] Forced TFF1 expression in MCF-7 and T47D breast cancer cells was associated with up-regulation of c-myc, CCND1 and CCNE1, and resulted in increased proliferation and survival, as well as enhanced migration and metastatic properties. Enhanced expression of several cyclins and c-myc also occurs through the Insulin-Like Growth Factor-1 (IGF-1) receptor system[36] known to stimulate breast cancer mitosis, anti-apoptosis and metastasis. ERα is critical in the activation of this pathway, inducing expression of several IGF-1 family members.[39,40] Re-expression of ERα in ER-negative MCF-7 breast cancer cell sub-lines also reactivated the IGF-1 pathway, which could be reversed with ERα antagonists.[41] IGF-1R/ERα crosstalk is also bi-directional, as IGF-1 induces transcriptional activity of ERα and increases expression of estrogen-inducible genes. In human breast cancer cells ERα activity could be blocked with IGF-binding protein-1.[42] In addition, stimulating MCF-7 cells with estrogens rapidly induces IGF-IR, EGFR and MAPK activation through non-genomic ER signalling. EGF-R was was demonstrated to act in a linear sequence downstream of the IGF-R pathway. Blocking this pathway diminished estrogen induced mitogenic and anti-apoptotic effects.[43] Furthermore, estrogens are potent apoptosis inhibitors, in contrast to anti-estrogens that have a profound pro-apoptotic effect. ERα enhances the expression of several anti-apoptotic genes, including Bcl-2 and $Bclx_L$.[44] Moreover, the before-mentioned increased TFF1 expression also downregulates the pro-apoptotic genes BBC3 (PUMA) and MDM2.[45]

While ERα skews cancer cells towards highly proliferative and apoptosis resistant tumour cells, RAR is typically referred to as tumour suppressive. Remarkably, ER and RAR counteract each other in cell cycle control and apoptosis pathways. *In vitro* studies have demonstrated that RAR ligands effectively repress proliferation in normal epithelial cells and ER positive breast cancer cells through G1 cell cycle arrest, associated with reduced cyclin D1 and -D3 expression.[46] Additionally, retinoic acid (RA) triggers cell cycle arrest through immediate early RAR targets including the cell cycle regulator Btg-2. Induction of Btg-2 by RA was accompanied by a marked decrease in cyclin D1 expression, hampering $G_1$- to S-phase transition.[47] Apart from cell cycle regulation, RAR also counteracts ER function in the apoptotic pathway. The anti-apoptotic protein Bcl-2 was clearly downregulated in RA induced apoptotic cells, as was survivin, another suppressor of apoptosis.[48,49] In addition, RA-induced apoptosis in MCF-7 cells was associated with an increased expression of several pro-apoptotic genes including caspase 7 and 9. Opposite to caspase 7, caspase 9 harbors a retinoic acid response element (RARE) and transcription is depended on RAR activation.[50]

It is clear from these functional studies that ER and RAR use similar pathways and even genes to exert opposite effects. In many studies breast cancer cells are usually treated with single ligands. Since physiologically cells are constantly triggered by multiple NR ligands, crosstalk between NR is inevitable. We need to learn much more about the physiological settings in which multiple NR ligands are around to determine the effect of their copresence on cell growth, -division and –death and the consequences for epithelial cell behaviour and breast cancer aetiology.

## CROSSTALK OF ER & RAR AT THE NUCLEAR RECEPTOR LEVEL

Crosstalk of NR has been suggested for years and may involve different steps in the pathway.[3] NR can share or compete for ligands, DNA response elements, or binding partners. They can also regulate the expression of other NR or behave as coregulators. Moreover, target gene products of a certain NR may affect the functionality or biosynthesis of other NR ligands. The consequences of crosstalk is that the expression of given set of target genes expected to be regulated by a given signalling pathway, is in fact dependent on the functioning of other signalling pathways.[51] Crosstalk between different pathways will ultimately lead to an appropriate response of cells in a certain condition.

In breast cancer research the crosstalk between RAR and ER has recently regained increasing attention. It is known for long that estrogens can induce RAR expression,[52,53] whereas RA induced RAR stimulation in breast cancer cells correlates with the decreased ERα expression.[54] In addition it has been shown that ER repressed the transcriptional activity of RAR/RXR mediated transcription and this could be overcome by the addition of RA.[55] Recently, three relevant research papers were published that provide novel insight in the mechanism of crosstalk between ER and RAR in breast cancer cells.[9,56,57]

Hua and colleagues[56] analyzed the genomic actions of RA signalling through RAR in MCF-7 breast cancer cells. By genome-wide mapping of RAR binding sites the authors found that RA signalling regulates the expression of many genes that are implicated in breast carcinogenesis. They also found extensive colocalization of RAR with ER DNA binding sites in the vicinity of genes that are antagonistically regulated by estrogen and RA. The majority of RAR binding sites were found in intronic or promoter-distal intergenic regions. As many ER and RAR binding sites appeared as neighbouring or even (partially) overlapping elements, the authors propose NR competition for the same closely spaced binding element as one mechanism for the antagonistic regulation of these genes upon RA treatment. Moreover, they demonstrated that FoxA1 and GATA3 binding coincided with RAR and ER binding, indicating that these transcription factors may also play an important role by coregulating ER and RAR on shared ER/RAR-binding elements. Hua *et al.*,[56] postulate that ER and RAR are the “Yin and Yang” for the genetic regulation of proliferation and survival that are promoted by ER and inhibited by RARs. The ER/RAR antagonism appears to regulate itself through cross regulatory loops between ER, RARs and their cofactors. This balanced control of gene expression regulates fundamental cellular processes that when dysregulated can lead to cancer.[56]

Ross-Innes and colleagues[57] also mapped the RAR-binding events in MCF-7 and did this in the presence of the ER-ligand estrogen instead of RA. Moreover, these authors were the first to succeed in mapping the endogenous RAR-binding events. Their data demonstrated that RAR and ER can be part of the same transcriptional complex. Using Re-ChIp and co-immunoprecipitation experiments they show that RAR/ER co-occupancy does occur in a cooperative manner in the presence of estrogen. Analysis of the genomic location of the RAR binding events revealed that most binding events of RAR occurred in intergenic regions. However, RAR could also bind to ER-binding regions and depended in half of these sites on the presence of ER. In contrast, ER binding to chromatin was independent of the presence of RAR; RAR rather functioned as a scaffold in the ER complex for maintaining cofactor interactions. Upon addition of RA, the estrogen-ER-dependent role for RAR could be inhibited and could have antagonistic effects on ER transcriptional activity similar to as what has been described by Hua *et al.*.[56] ChIP analysis of hormone-depleted MCF-7 cells revealed that in estrogen treated cells the co-presence of RA did not perturb binding of either receptor. The co-presence of RA rather diminished the amount of the cofactors p300 and TRAP220 that are essential for effective transcription at ER/RAR regulated genes. The authors postulate two independent functions for RAR. In response to natural ligands such as RA it exerts its classical role as heterodimeric partner of RXR while during estrogen treatment it can function as an ER-associated protein required for maintaining cofactor interactions. Therefore, any shift between the classic and novel pathways may influence ER function in breast cancer cells. RA promotes the classic role of RAR at the cost of Estrogen-ER function.[57]

The recent identification of a novel NR modulator, termed DC-SCRIPT, suggest that ER/RAR crosstalk might not only take place at the NR level, but also on the coregulator level. The 11 zinc fingers containing protein DC-SCRIPT also contains a functional CtBP1 binding motif[10] and a putative LxxLL NR interaction motif. It has been shown that DC-SCRIPT can interact with multiple transcription regulatory proteins such as CtBP1, RIP140 and HDAC1, 3 and 6, suggesting that DC-SCRIPT is present in very large multiprotein complexes known to be involved in NR regulation.[9,58-60] DC-SCRIPT was shown to interact with multiple NR, including ER and RAR. DC-SCRIPT was able to repress transcription mediated by ER and surprisingly enhanced transcription mediated by RAR. Interestingly, exogenous DC-SCRIPT in MCF-7 was shown to opposingly regulate transcription mediated by multiple NR at the same time. In the presence of multiple NR ligands, DC-SCRIPT was able, in a single cell, to repress transcription mediated by the type I NR PR and activate transcription mediated by the type II receptor RAR. To our knowledge, there are currently no coregulators known that have such a distinct effect on type I and type II mediated transcription. So far, studies investigating crosstalk between ER and RAR have mostly been performed in MCF-7. As this cell line and all other cell lines tested so far are essentially negative for DC-SCRIPT, in contrast to breast epithelial cells, the effect of DC-SCRIPT on the estrogen and RA signalling pathways in breast cancer cells remains to be investigated. This may shed novel insight into the crosstalk between ER and RAR.

Collectively, these novel data are indicative for the high level of complexity in transcriptional crosstalk between ERα, RAR and DC-SCRIPT (see figure 1). Upon estrogen or RA stimulation, the corresponding NR can exert their classical role or behave as a coregulator or cofactor of the other. Depending on cellular context, expression levels of DC-SCRIPT, different NR and their coregulators and the presence or absence of (multiple) ligands, cells are skewed to a certain genetic program. Extensive crosstalk between NR is essential to enable cells to fine-tune their response and promptly adapt to different environmental situations.

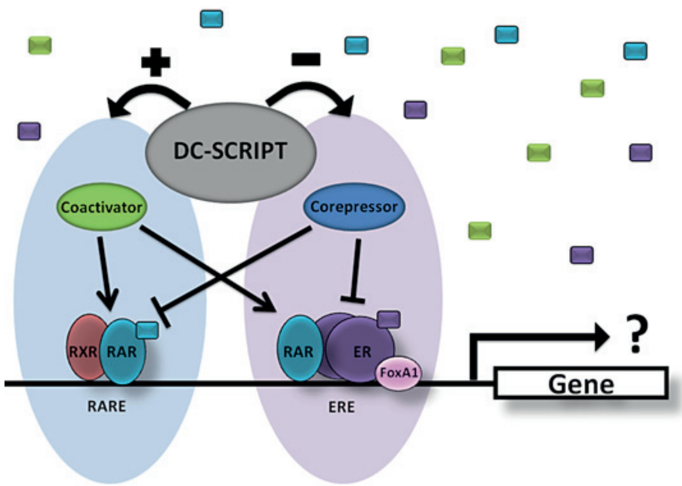


***Figure 1. Transcriptional crosstalk between DC-SCRIPT, RAR and ER***
*Hypothesized model of the induction of an estrogen and retinoid responsive gene. RAR and ER binding sites have been shown to be in close proximity. RAR can function as a scaffold for ER binding or can act in its more classical role as heterodimeric partner of RXR. FOXA1 acts as a pioneering factor for ER binding. Both coactivators and corepressors regulate RAR and ER function. DC-SCRIPT can exert a dual role in this regulation. It represses ER function, and stimulates RAR function. The available ligands present will induce the crosstalk between DC-SCRIPT, NR, its regulators/cofactors and will ultimately lead to an appropriate cell response.*

## NUCLEAR RECEPTORS, COREGULATORS AND BREAST CANCER

The incidence of breast cancer is known to be affected by the hormonal milieu breast cells experience over time. Important hormone-related risk factors for breast cancer include early menarche, nulliparity or late age at first birth and late menopause.[61] The type I hormone-inducible NR play an important role in the tumourigenesis of breast cancer. Breast tumour tissue expressing the type I NR, ER and/or PR, exhibit a well-differentiated phenotype indicating a good prognosis for the patient. Large scale expression profiling studies to classify tumours[62-64] confirmed that ER/PR positive tumours are distinct from other breast tumour types on the basis of their gene expression pattern.[63,65-67] Within the ER/PR positive tumours, two distinct types could be distinguished showing a different prognosis; the histologically low-grade luminal A subtype and the more often high-grade luminal B subtype.[63,65,68] These data show the importance of ER/PR status for prognosticating of breast tumours. In addition, ER/PR status is predictive for tamoxifen sensitivity, a highly effective anti-estrogen treatment for breast cancer patients.[69,70] These findings emphasize the importance of molecular signatures but also indicate that additional markers are needed to enhance the understanding of breast cancer etiology and improve prognosis and treatment selection.

Besides the type I NR, also type II NR have clinical implications in breast cancer. Both low vitamin D ingestion and low circulating 25-hydroxyvitamin D3 levels are associated with increased breast cancer risk, indicating a protective role of the type II NR VDR in breast cancer tumourigenesis.[71] RARα mRNA expression has recently shown to be associated with a good prognosis in endocrine treated breast cancer patients.[57] Moreover, Hua *et al.*[56] were able to define a RA-induced gene expression profile associated with a favourable prognosis. Furthermore, increased expression of another type II NR, PPARγ, in the primary tumour also correlated with improved survival.[72] Collectively, these data imply that through their anti-proliferative and pro-apoptotic effects the type II NR and their target genes are correlated with a positive clinical outcome. Currently, no adjuvant treatments directed at type II NR are routinely available for breast cancer. Stimulation of the NR RAR/RXR and PPAR/RXR has been explored[73,74] and synthetic RAR ligands have been shown to reduce second breast cancers.[75] However, so far efficacy has been limited because of retinoic acid resistance acquired during cancer development.[76,77] Therefore, the effect of RA in oncology has so far been limited. The only successful application of RA treatment is in APL (acute promyelocytic leukemia) patients[78] where it drives cell differentiation. Whether RA has a similar effect in breast cancer cells is not known.

Consistent with the finding that both type I and type II NR function are dependent on coregulators, several groups have reported that NR co-regulator function is highly relevant for prognosis and endocrine therapy sensitivity in breast cancer as well.[7,79] A well-studied example with relevance to breast cancer is the ER coactivator Amplified in Breast Cancer-1 (AIB1 or SRC3[80]). Overexpression of AIB1 results in increased ER activity and is generally associated with a poor prognosis in breast cancer.[81-83] Some studies, however, do find a relation between AIB1 and a good prognosis.[84,85] As AIB1, like other coactivators, is positively correlated with ER status[86] it might be indirectly associated with a good prognosis through association with the ER-positive luminal subtype of breast cancer. Possibly, within this ER-positive luminal subtype, ER coactivators might be associated with a relatively poor prognosis.[87] The ambivalent prognostic value of AIB1 in breast cancer might thus be explained by the fact that these ER/PR positive patients are most often treated with adjuvant tamoxifen and that AIB1 is associated with tamoxifen resistance.[82,85] Furthermore, coexpression of growth factor receptors[82,85] or the subcellular localization of AIB1[84] has been suggested to affect its prognostic value in breast cancer. Other cofactors that play a critical role in hormonal signalling, including the coactivators FOXA1 and GATA3, appear to be markers of luminal A breast cancers. Expression of these factors has shown to be related to good prognosis and to endocrine therapy sensitivity.[88] Another coactivator whose function in determining breast cancer prognosis is unclear, is steroid receptor coactivator 1 (SRC1 or NCOA1). SRC1 was originally described as denoting a good response to tamoxifen treatment,[89] then found to be associated with poor survival and poor response to tamoxifen.[87,90,91] More recently it has been found again to be associated with a good prognosis.[86] For SRC1, the coexpression of other transcription factors such as Ets-1 and Ets-2 might be important in determining its effect on prognosis.[87,91] Corepressors like NR corepressor 1 (NCOR1) and -2 (NCOR2 or SMRT) were also found to be associated with a good response to tamoxifen,[87,91,92] although these studies did not include a non-treated cohort to distinguish a prognostic from predictive biomarker. Conversely, NCOR2 has also been shown to be associated with poor survival in a large breast cancer cohort.[86]

Approximately 300 coregulators have now been identified, of which at least 165 have so far been directly associated with human diseases.[33,93,94] It will be important to further delineate the positive or negative effects on the transcription factor pathways involved. Of note, many coregulators are able to bind to, and regulate, both type I and type II NR.[15] Remarkably, most -if not all- coregulators have either a stimulatory or inhibitory effect on both type I and type II NR, with the notable exception of the newly discovered NR coregulator DC-SCRIPT.[9] DC-SCRIPT acts as a unique coregulator of multiple NR having opposite effects on type I vs type II NR. Recently, Ansems and colleagues[9] evaluated DC-SCRIPT expression in breast tissue and showed that DC-SCRIPT is expressed in breast ductal epithelial cells. In addition it was demonstrated that breast tumours expressed lower levels of DC-SCRIPT than normal breast tissue from the same patient. Moreover, quantification of DC-SCRIPT mRNA expression in three cohorts of breast cancer patients revealed that DC-SCRIPT mRNA expression is an independent prognostic factor for good survival for breast cancer patients with ER- and/or PR-positive tumours.[9] The prognostic significance was maintained in a cohort of tamoxifen treated patients. Together with our finding that DC-SCRIPT inhibits cell growth of breast carcinoma cells our data suggests that DC-SCRIPT can act as a tumour suppressor in breast cancer development (see figure 2). This makes DC-SCRIPT an attractive target for NR coregulator specific therapy for either breast cancer prevention or adjuvant therapy.

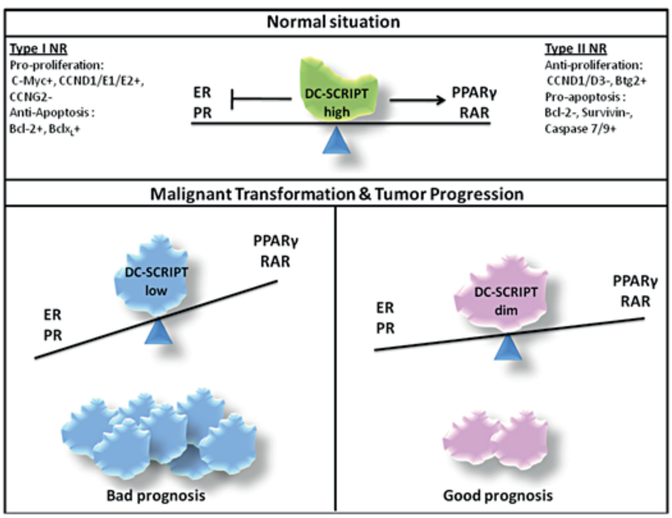


***Figure 2. Balance of Nuclear Receptor function by DC-SCRIPT expression***

*Hypothesized model of DC-SCRIPT function in ER+ breast epithelial cells. DC-SCRIPT represses the activity of the pro-tumourigenic type I NR ERα and PR and conversely enhances the anti-tumourigenic type II NR PPARγ and RARα, thereby actively regulating the NR balance. During malignant transformation and tumour progression, higher DC-SCRIPT expression leads to a more balanced NR function resulting in a better prognosis, whereas low expression of DC-SCRIPT results in an imbalance in NR function and to a bad prognosis.*

## FUTURE PERSPECTIVES

Molecular and functional studies on individual NR are at the forefront of life science research. They have led to novel insights into genome-wide transcription factor binding patterns and gene regulation. An emerging challenge is to unravel how NR family members act in concert to regulate complex cellular processes in tissues where multiple NR ligands are present. The examples presented in this review, the crosstalk between ER and RAR and the action of the recently identified bi-functional transcription modulator DC-SCRIPT, only provide initial insights in these processes. It will be interesting to further unravel both the genomic as well as non-genomic effects of NR on cellular behaviour and how they relate to malignant transformation. Regarding its function as a type I and type II NR coregulator and its prognostic relevance in breast cancer, it will be rewarding to investigate the impact of DC-SCRIPT and ER/RAR crosstalk in other hormone sensitive tumours like prostate and endometrium cancer. An important question that remains to be answered is how DC-SCRIPT genotype and expression levels will affect the cell's response to hormones, vitamins and metabolites and whether DC-SCRIPT malfunction is sufficient to act as a priming event in malignant transformation.

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# Chapter 7

## Summary and discussion

## TRANSCRIPTION REGULATION

Regulation of gene expression by transcription factors (TFs) is essential for proper cell differentiation, proliferation and function. Aberrations in this process through misregulation or dysfunction of TFs cause a wide variety of human diseases, including developmental disorders and cancer.[1] Activation of gene transcription occurs via Histone Acetylation by Histone Acetyl Transferases (HATs), inducing chromatin opening and recruitment of the transcription initiation complex for RNA polymerization. In contrast, gene repression involves the recruitment of Histone DeACetylases (HDAC), that cause chromatin condensation and thereby prevent binding of RNA polymerases. Classically, TFs are thought to function as individuals, regulating a particular subset of genes via factor specific mechanisms. Recent insights from genome-wide profiling studies, however, suggest a more complex reality in transcription regulation. Instead of targeting specific gene subsets, TFs were found to bind thousands of places throughout the genome, often located outside proximal promoter regions and lacking factor specific consensus motifs. These sites regulate transcription of distal genes via cooperative binding of TFs. This implies that each TF can exert multiple roles depending on the binding site and the recruitment of other factors. Moreover, not only do these factors bind directly to the DNA motifs, they can also engage in protein-protein interactions, hence functioning as transcriptional co-regulators.[2]

In this thesis we investigated the transcriptional co-regulatory properties of DC-SCRIPT (DC-Specific transCRIPT) in both dendritic cell (DC) and tumor cell biology, and found both repressive and activating roles. In this chapter we further discuss our findings, and provide insight into the potential implications.

## DC-SCRIPT AS TRANSCRIPTION (CO-)REGULATOR

Previous studies have identified DC-SCRIPT as a putative transcription factor that is specifically expressed by DCs within the immune system. Its 11 zinc-fingers were suggested to function in DNA binding, allowing transcriptional regulation of specific genes.[3,4] Albeit no direct target genes for DC-SCRIPT have been identified to date, a regulatory role in gene expression was indeed observed. In **chapter 3** we identified DC-SCRIPT to be an important factor in the control of IL-10 expression,[5] whereas **chapter 4** (S. Hontelez *et al.*, submitted) and **chapter 5**[6] show a role in NR co-regulation.

In general, transcription regulators can be either ubiquitously (>30 tissues), or specifically expressed (<3 tissues), suggesting very broad, or very specific functions.[1]

DC-SCRIPT apparently falls into the second category, as its expression was only detected in DCs and epithelial cells. This suggests that regulation of gene expression by DC-SCRIPT is particularly important in these cell subsets. Indeed, transformation of epithelial cells towards tumor cells is accompanied by loss, or severe reduction of DC-SCRIPT levels, and knock-down in DCs results in impaired DC maturation, illustrating the importance of DC-SCRIPT expression in these cells. DC-SCRIPT possibly exerts overlapping roles in both cell types, however, since DCs and epithelial cells have a very different biology and function, DC-SCRIPT likely controls the expression of multiple gene subsets that play different roles in these cells. It would therefore be interesting to further investigate how DC-SCRIPT functions in these different cell types.

*NR co-regulator*

Wide-spread control of gene expression is a known feature of the Nuclear Receptor superfamily of TFs. In contrast to DC-SCRIPT, many NRs are ubiquitously expressed and are involved in a plethora of cellular processes and functions. However, cell- and tissue specific functions have also been described, and are based on variable expression of NR isoforms, post-translation modifications and cell specific expression of NR co-regulators.[7,8] Interestingly, DC-SCRIPT has previously been identified as a NR co-regulator in epithelial breast carcinoma cells.[9] In **chapter 5** of this thesis we further investigated the role of DC-SCRIPT as NR co-regulator,[6] and we have extend its regulatory function to DC biology in **chapter 4** (S. Hontelez *et al.*, submitted).

As NR co-regulator, DC-SCRIPT was found to function both as co-repressor and co-activator, depending on the NR. In general, DC-SCRIPT was shown to co-repress Type I NR transcriptional activity, whereas co-activation was primarily detected on Type II NR mediated transcription. Transcriptional assays demonstrated repression on Type I NR ERα, PR-B, GR and AR, and activation of Type II NR RARα/RXR, PPARγ and VDR function. Besides co-repression of the Type I NR ERα, PR-B, GR and AR, DC-SCRIPT was also shown to stimulate GR mediated transcription, depending on the promoter (discussed in *Promoter specific effects*, page 138). Furthermore, activation of endogenous VDR target genes, and repression of endogenous AR and GR target genes were demonstrated, suggesting an physiologically relevant mechanism. Modulation of other NR target genes could, however, not be demonstrated, possibly due to differences in promoter and cell context.

As NR co-regulator, DC-SCRIPT was found to interact with both Type I and Type II NRs. Co-Immunoprecipitation experiments from cell-lines demonstrated exogenous DC-SCRIPT to co-exist in protein complexes with exogenous Type I NRs ERα, PR-B, AR or GR, or Type II NRs RARα/RXR, VDR and PPARγ. Endogenous interactions could only be examined in DCs, the only DC-SCRIPT positive cell that can be obtained in sufficient numbers. However, despite efficient immunoprecipitation of DC-SCRIPT from DCs, co-presence of NRs in the IP fractions could not be discerned. DCs express 20 different NRs that all possibly contain DC-SCRIPT in their repressor or activator complexes. Since the IP of DC-SCRIPT only pulls-down a fraction of the expressed protein, enrichment of a specific NR via DC-SCRIPT IP may be very difficult. In addition, endogenous unbound DC-SCRIPT may possibly bind more efficiently to the beads, and is therefore more likely to be purified. In transient expression systems, on the other hand, both DC-SCRIPT and NRs are over-expressed, what may result in a higher occurrence and stability of complex formation. Experiments that aim the Co-IP of DC-SCRIPT by IP of a NR face the same problem, as NRs are also engaged in many different protein complexes. Recently, Co-IP of endogenous NRs with exogenous DC-SCRIPT did show the interaction with AR in prostate carcinoma derived cell-lines.[10] Furthermore, Co-IP findings are supported by functional data that demonstrate the effect of DC-SCRIPT expression on NR target gene expression. Proper assessment of endogenous interactions between DC-SCRIPT and NRs requires a more sensitive approach, such as Mass spectrometry (MS). This also importantly allows for comparative analysis between DCs and hormone responsive carcinoma cell-lines.

In exogenous settings, the co-presence of NRs in DC-SCRIPT protein complexes was not dependent on stimulation with the respective NR ligands. In addition, the LxxLL domain was found to be expendable, and the interactions were not detected in Yeast-2-Hybrid assays, suggesting indirect binding. In contrast, previously published Yeast-2-Hybrid data did show a direct interaction between DC-SCRIPT and ERα.[9] This discrepancy could originate from the cDNA library and yeast strains used in both studies. In our Yeast-2-Hybrid assays we used a DC-derived cDNA library and the YGHI yeast strain.[4] Lopez-Garcia *et al*. (2006) on the other hand, employed a human placental cDNA expression library in the PL1α yeast strain, containing an integrated estrogen-responsive URA3 gene. This possibly provides a platform for more efficient interactions between DC-SCRIPT and ERα, suggesting that direct binding is possible on some target gene promoters. However, binding of DC-SCRIPT to the promoter independent of ERα cannot be completely excluded in this system.

The co-presence of DC-SCRIPT in NR protein complexes likely depends on interaction with other proteins within this complex. Indeed, we previously revealed a direct interaction between DC-SCRIPT and CtBP1 (C-terminal Binding Protein 1) using Yeast-2-Hybrid assays.[4] Furthermore, DC-SCRIPT was previously shown to bind Receptor Interacting Protein 140 (RIP140) and HDAC1, -3 and -6.[9] Both CtBP1 and RIP140 function as transcriptional co-repressors and recruit HDACs to the promoter bound complex. Hence DC-SCRIPT is suggestively present in large co-repressor complexes, preventing NR mediated transcription. This fits with the ligand independent nature of the described NR interactions, since co-repressor complexes are usually associated with NR in the absence of ligand, or in the presence of antagonists. Importantly though, transcriptional assays demonstrate co-repression of Type I NR in the presence of their ligands, suggesting an central role for DC-SCRIPT as NR co-repressor. We have, however, also demonstrated DC-SCRIPT to function as NR co-activator, enhancing Type II NR. NR co-activators generally recruit other co-activator proteins that allow for chromatin remodeling, histone acetylation and recruitment of the RNA polymerase II complex.[7] Assessing the role of DC-SCRIPT in the recruitment of these protein complexes using Co-IP and/or Mass Spectrometry would increase our insight into the co-activator mechanisms employed by DC-SCRIPT.

*Promoter specific effects*

It is well known that most TFs differently modulate target genes. Co-factors, however, have also been demonstrated to have divergent effects on gene transcription. NR co-activators have been shown to repress gene expression in some cases,[11] whereas gene activation was shown by co-repressors.[12] This suggests that co-regulator action is variable depending on the gene, cell, and signaling context.[13] The DC-SCRIPT interacting co-repressor CtBP1 has also been shown to exert more than one function.[14] In this thesis, we have demonstrated multiple roles for DC-SCRIPT in transcription regulation, acting as co-repressor or as co-activator, depending on the NR. This variation in function also occurs in the regulation of a single NR, the GR. In addition to its function as GR co-repressor on the MMTV promoter, DC-SCRIPT was also observed to function as GR co-activator when the MMTV HRE sequences were isolated from their context and placed in a minimal promoter (figure 1**,** unpublished data).

Various mechanisms that have been described to modulate TF function, could also potentially affect the regulatory role of co-factors such as DC-SCRIPT. These include (i) differences in TF DNA binding sequence (ii) interaction with other TFs or regulatory proteins, (iii) post-translational modifications and (iv) multifunctional protein domains (e.g. zinc fingers). Many TFs have been shown to bind multiple transcription regulatory elements, however, the TF DNA binding sequences often differ between these sites. For the Type I NR Glucocorticoid Receptor (GR) it has been shown that single base pair differences in the Glucocorticoid Responsive Elements (GREs, the DNA binding sites for GR) already affect the regulatory activity of GR.

Binding to these different GREs causes variations in GR conformation and alters co-factor recruitment. A similar effect might be true for co-factors such as DC-SCRIPT. The binding properties of DC-SCRIPT possibly differ between TFs, or between different conformations of the same TF, affecting the subsequent recruitment of other co-factors. Interestingly, we indeed found that DC-SCRIPT exerts both roles on the same NR, the glucocorticoid receptor, depending on the promoter (figure 1).

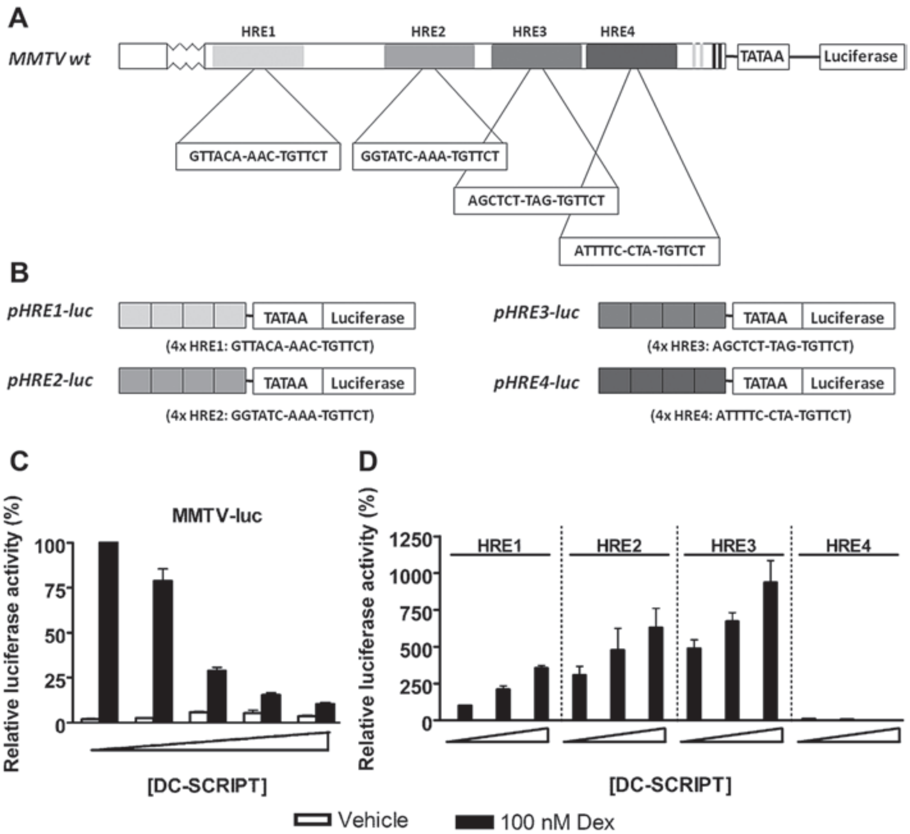


***Figure 1. DC-SCRIPT function on isolated HRE sequences from MMTV-luc***

*Schematic view of the (A) luciferase reporter plasmid MMTVwt displaying the four HRE sequences, (B) and the luciferase reporter plasmids HRE1-4-luc where the 4x consensus GRE sequence from GRE-luc is replaced by 4x repeat of the HRE1, 2, 3 or 4 sequence from MMTVwt. (C-D) Hep3B cells were co-transfected with the firefly luciferase reporter plasmids (C) MMTV-luc, (D) HRE1-luc, HRE2-luc, HRE3-luc or HRE4-luc, the expression plasmid for GR and increasing amounts of the expression plasmid for DC-SCRIPT. Cells were stimulated with vehicle (white bars) or 100 nM Dexamethasone (black bars) for 24 hrs. Luciferase activity is displayed relative to luciferase production upon Dexamethasone stimulation in the presence of (C) MMTV-luc or (D) HRE1-luc and in the absence of DC-SCRIPT.*

Besides the TF binding site, also the promoter context plays an important role. Multiple TFs can target a specific transcription regulatory site and catalyze or inhibit the recruitment of other factors. The function of each TF or co-factor might therefore differ between promoters, depending on the present TF community.[2] DC-SCRIPT function on GR, however, was not affected by deletion of transcriptional regulatory elements in the MMTV promoter. This suggests that binding of other factors does not affect DC-SCRIPT function in these conditions. In contrast, isolation of the MMTV Hormone Responsive Elements (HREs) from their context, in experiments where the GRE consensus sequence in the minimal promoter was replaced with one of the MMTV HRE sequences (figure 1), did affect DC-SCRIPT function. In these settings, DC-SCRIPT was found to function as GR co-activator, enhancing GR mediated gene transcription. These data therefore suggest an important role for the promoter context.

Since these results were obtained from transcription assays, it is important to assess the physiological relevance by testing the effect of DC-SCRIPT on endogenous target genes. In human moDCs, the endogenous Glucocorticoid Inducible Leucine Zipper (GILZ) was shown to be downregulated by DC-SCRIPT. Identification of other GR targets affected by DC-SCRIPT proved to be difficult (data not shown). GR-ligand dependent upregulation could not be detected for eight out of 10 tested target genes that were previously identified in other cell-types to be GC responsive. This could possibly be explained with cell-type specific differences such as differential expression of the repressive GRα-D and GRβ isoforms. Increased expression upon GR activation was detected for FKBP5 and PTX3, however no effect of DC-SCRIPT silencing could be discerned, possibly due to the GRE sequence or context. Furthermore, also functional redundancy and incomplete silencing could play a role. Although DC-SCRIPT protein levels are greatly reduced upon silencing, some expression could still be detected, which may be sufficient for the repression of certain GR target genes in moDCs.

Another important mechanism that affects TF and co-factor function are post-translational modifications (PTMs), which modulate DNA and protein binding properties, subcellular localization, stability and activity. Combinatorial usage of these rapid and reversible modifications provide immense regulatory possibilities that can be appropriately adjusted to the situation and cell context. These modifications include phosphorylation, glycosylation, SUMOylation, acetylation and ubiquitinylation.[15] NRs are known to be modulated by PTMs,[8,16] possibly affecting DC-SCRIPT binding. In addition, DC-SCRIPT itself might also be regulated by PTMs, since putative phosphorylation-, SUMOylation and *N*-glycosylation sites have also been detected in its amino acid sequence.[4] Future identification and functional analysis of PTM sites in DC-SCRIPT requires Mass Spectrometry and loss-of-function studies through mutations of specific PTM sites.

Intrinsic protein properties of DC-SCRIPT might also account for the diverse transcriptional regulation. An important domain in the amino acid sequence is the zinc finger region, containing 11 Cys2-His2-type zinc fingers. This type of zinc fingers is well known for its DNA-binding activity, suggesting DC-SCRIPT directly targets promoter/ enhancer DNA of specific genes. However, these zinc fingers are also implicated in protein-RNA and protein-protein interactions. Interestingly, alternate usage of zinc fingers allows a protein to engage in different interactions.[17,18] Combinations of the 11 zinc fingers present in DC-SCRIPT can therefore be attributed for binding different TFs, co-factors and DNA sequences. In addition, we found different domains of DC-SCRIPT to be important in NR co-regulation. For instance, co-repression of ER requires the presence of the proline-, zinc- and acidic domain, however, for the repression of GR and AR dependent transcription, the proline region was found to be sufficient (unpublished data). Likewise, all three domains are required for co-activation of PPARγ mediated transcription, whereas only the acidic domain was necessary for stimulation of RARα/RXR transcriptional activity. This illustrates that the zinc fingers are not always necessary, and that DC-SCRIPT utilizes different domains depending on the situation. Expression studies involving ChIP-seq and RNA sequencing or exon expression profiling in cells expressing *wt* DC-SCRIPT or DC-SCRIPT deletion mutants is necessary to identify regulated genes and the involved domains.

## DC-SCRIPT IN DENDRITIC CELLS

Dendritic cells play an essential role in our immune system. They induce and modulated immune responses against pathogens and tumors, while protecting the host from auto-immunity.[19] The preferential expression of DC-SCRIPT in DCs within the immune system suggests a specialized role in these cells.[4] In **chapter 3** we have, to our knowledge, for the first time characterized endogenous DC-SCRIPT protein expression in different DC subsets both *in vitro* and *in vivo.*[5] Furthermore, we demonstrated an important role for DC-SCRIPT in DC maturation. Silencing of DC-SCRIPT expression induced IL-10 secretion upon TLR4 and TLR7/8 stimulation, which subsequently impaired IL-12 production. In **chapter 4** we further elucidated the function of DC-SCRIPT as NR co-regulator in DCs and demonstrated co-repression on the GR dependent expression of GILZ (S. Hontelez *et al.*, submitted). Here, we further discuss the function and implications of DC-SCRIPT expression in human DCs.

*DC differentiation*

Transcription factors have been shown to play an important role in DC development. Some factors are involved in the differentiation of all DC-subsets, whereas other factors are uniquely found in the development of a specific subset. The TFs Ikaros, PU.1 and Gfi1 for example are found in all DC subsets, and loss-of-function causes the ablation of most DC populations in mice. On the other hand, factors like IRF2, -4 and -8, Id2, Spi-B and RelB have specialized functions in the differentiation of either cDCs, pDCs or LCs.[20]

All DC subsets tested to date, including myeloid DCs (MDCs), plasmacytoid DCs (PDCs) and Langerhans cells (LCs) were found positive for DC-SCRIPT protein expression. *In vitro* differentiation of DCs from monocytes requires stimulation with IL-4 and GM-CSF.[21] Remarkably, expression of DC-SCRIPT protein was detected within 4 hours upon differentiation from monocytes towards DCs, and was found to be largely dependent on IL-4 stimulation. Protein expression levels further increased during development, with maximum levels detected in fully differentiated immature and mature DCs. Interestingly, previous analysis of the DC-SCRIPT promoter demonstrated putative binding sites for DC specific TFs PU.1, Spi-B, c-Rel, Ikaros 2 and Gfi. In addition, also other TFs with an important function in DC biology suggestively bind the DC-SCRIPT promoter, including GATA-1 and the TF complexes NF-κB and AP-1.[3]

These findings suggest that DC-SCRIPT is an important factor in the development and function of different DC subsets. Its precise function in DC differentiation is, however, currently unknown. Analysis of the DC-SCRIPT promoter, knock-down studies with DC-SCRIPT targeting siRNAs, and the use of DC-SCRIPT knock-out mice are therefore important to reveal the significance of DC-SCRIPT in DC development.

*DC maturation*

DCs can mature into different states depending on the pathogen and environmental stimuli. This diversity allows DCs to differentially affect T-cell polarization thereby tailoring the immune response to the present pathogens. Generally, mature DCs can be immunostimulatory, or immunosuppressive. In **chapter 3** and **4** of this thesis we have uncovered an important regulatory role for DC-SCRIPT in the expression of IL-10[5] and GILZ (S. Hontelez *et al.*, submitted), two well known hallmarks of immunosuppressive, or tolerogenic DCs (tolDCs) (figure 2). In moDC, DC-SCRIPT was shown to repress the expression of both genes, and was therefore suggested to skew DC maturation towards immunity. In line with these results, DC-SCRIPT mRNA levels were significantly increased in immunostimulatory DC, albeit no differences at protein level could be discerned. Possibly, LPS stimulation increases turnover of

DC-SCRIPT protein during DC maturation. To compensate for lost protein expression DCs increase transcription of the *DC-SCRIPT* gene, thereby maintaining equal protein expression levels. DC-SCRIPT proteins levels in tolDCs were comparable to immature and mature DCs, therefore other mechanisms regulating DC-SCRIPT function upon GR activation must be in place, such as PTM or binding of chaperone proteins.

When DCs mature in the absence of danger signals, or in the presence of immunosuppressive stimuli, they turn into tolDCs. These DCs express low levels of co-stimulatory molecules, and therefore suppress T-cell proliferation. In addition, secretion of the anti-inflammatory cytokine IL-10 is enhanced, which could trigger Treg polarization.[22] DC-SCRIPT knock down in moDCs increased IL-10 secretion which subsequently impaired production of the pro-inflammatory cytokine IL-12 (figure 2). Other pro-inflammatory molecules such as cytokines IL-6 and TNF, and receptors CD80, -83- and -86 (CD86 data not shown), however, were not affected. This suggests that generation of tolDCs requires more than just the repression of DC-SCRIPT signaling.

Alternatively, DC-SCRIPT down-regulation may also be important for the maturation of Th17-polarizing DCs. Besides IL-23, IL-6 and TNF, generation of Th17 responses also requires inhibition of Th1 and Th2 responses.[23] Th1 responses inhibit Th17 differentiation, therefore an environment with high IL-10 and limited IL-12 secretion will restrain Th1 differentiation and favor Th17 skewing. Th17 cells have been shown to function in anti-fungal responses, and recognition of *C. albicans*, skews DC maturation towards Th17-polarizing DCs.[24] In **chapter 2** we describe the crosstalk between PRRs mediating the detection of fungal antigens.[25] Interestingly, collaboration between Dectin-1, TLR2/6 and Galectin-3 receptors results in cytokine secretion profiles similar to those seen upon DC-SCRIPT knock-down.

One way of enhanced and prolonged IL-10 transcription in DCs is NF-κB acetylation.[26] In DCs, IL-10 expression greatly depends on NF-κB activation.[27] Interestingly, we have recently obtained preliminary data that suggest a potential effect of DC-SCRIPT on NF-κB dependent transcription. Transcription assays with inducible NF-κB actelylation, as well as the use of specific signaling pathway blockers in DC-SCRIPT knock-down experiments would provide important information on the putative role of DC-SCRIPT on NF-κB transcription regulation.

In **chapter 4** we show that DC-SCRIPT also inhibits the expression of another important molecule in tolDCs, GILZ (glucocorticoid induced leucine zipper) (S. Hontelez *et al*., submitted). TolDCs can be generated through stimulation with glucocorticoids (GCs) prior to DC maturation.[28] GCs activate GR, a Type I NR with profound immunosuppressive properties.[29] Within DCs, GR activation impairs STAT, NF-κB, AP-1, 14-3-3, Raf-1 and Ras signaling, thereby preventing up-regulation of co-stimulatory molecules upon DC maturation. Expression of the GR target gene GILZ is induced upon GC stimulation, and serves an important role in mediating the immunosuppressive effects by GR. In fact, expression of GILZ in the absence of GR activation was demonstrated to be sufficient for the generation of tolDCs, whereas silencing GILZ expression prevented tolerance.[30] In DCs, silencing of DC-SCRIPT resulted in elevated, GR dependent, GILZ expression, suggesting DC-SCRIPT inhibits GR transcriptional activity on the GILZ promoter (figure 2). In the absence of GCs, also slight expression of GILZ was detected in DC-SCRIPT knock-down DCs, but not in control DCs. This could be an effect of the increased IL-10 expression in these cells, as IL-10 also induces the expression of GILZ in DCs.[31]

Together, these data imply an important role for DC-SCRIPT during DC maturation, favoring immunity over tolerance by repressing both IL-10 and GILZ expression.

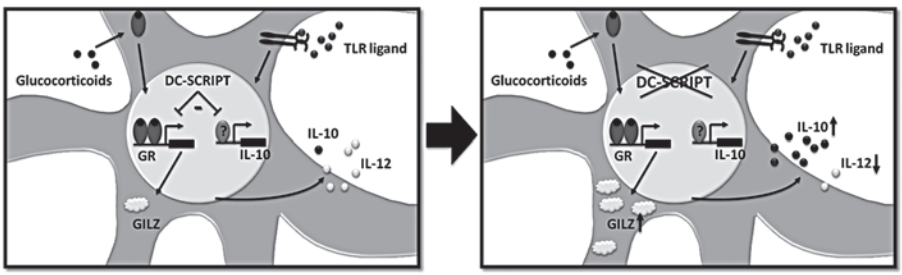


***Figure 2. DC-SCRIPT regulates IL-10 and GILZ expression in DCs.*** *In the presence of DC-SCRIPT, the anti-inflammatory cytokine IL-10 is expressed at low levels, while the pro-inflammatory cytokine IL-12 is abundantly produced. Knock-down of DC-SCRIPT results in high IL-10 secretion upon TLR stimulation, which consequently inhibits IL-12 secretion by these cells. In addition, DC-SCRIPT in DCs represses ligand depended and GR mediated transcription of GILZ, possibly preventing oversensitivity of DCs to the tolerogenic effects of glucocorticoids. Knock-down of DC-SCRIPT relieves repression on GR, resulting in an significant increase in GILZ expression levels, which has been shown to mediate the generation of tolDCs.*

## DC-SCRIPT in tumor cells

Next to DCs, also breast epithelial cells were found positive for DC-SCRIPT expression. Malignant transformation of these cells leads to breast cancer, and is accompanied by decreased, or complete loss of DC-SCRIPT expression. NR signaling plays an important role in the development and malignancy of this cancer.[32] In **chapter 5** we have demonstrated DC-SCRIPT to be involved in regulation of NR activity in these cells, balancing the activity of proliferative and anti-proliferative actions of both Type I and Type II NRs.[6] Indeed, this balance is important for proper NR crosstalk, and the maintenance of a healthy cell phenotype, which we further discussed in **chapter 6**.[33]

*Prognostic marker*

Despite the major improvements in detection and treatment, breast cancer is still one of the main causes of cancer death in women.[34] Different prognostic markers are currently used in the clinic to predict treatment efficacy, tumor recurrence and patient survival. Classically, these include pathological features, such as tumor size, histological subtype and grade and lymph node metastases. Molecular- and genetic markers have also been identified, with a central role for NR and their co-regulators.[35] In particular the Type I NRs ER and PR are well established markers, and their expression is positively correlated with a good prognosis.[36] Additionally, also the Type II NRs RAR/RXR and PPAR, as well as various NR co-regulators, have been described as important biomarkers for breast carcinoma.[32]

In **chapter 5** we have characterized DC-SCRIPT as a prognostic marker in breast carcinoma, concomitant with its function as NR co-regulator.[6] DC-SCRIPT was found to be a strong and independent prognostic marker for disease-free survival of breast cancer patients with ER- and/or PR-positive tumors. These results were based on 3 relatively small cohorts from Nijmegen, and were recently confirmed by a validation study on a independent cohort of 1505 primary breast cancers from Rotterdam. The latter showed DC-SCRIPT to be a predictive factor for the occurrence of distant metastasis in patients that did not receive adjuvant therapy, independent of other currently used prognostic markers.[37] In another recent study, DC-SCRIPT was also implicated in prostate cancer. This hormone responsive carcinoma develops from the cells that align the ducts of the prostate glands.[38] DC-SCRIPT expression was observed in morphologically normal prostate glands and infiltrating immune cells with IHC staining. As with breast epithelial cells, DC-SCRIPT expression in the prostate was lost in malignant prostate epithelial tissue and prostate carcinoma cell lines.[10]

Collectively, these data suggest an important role for DC-SCRIPT in preventing development and malignant transformation of hormone responsive tumors. Since DC-SCRIPT has been shown to regulate multiple NRs, it is likely to affect other epithelial derived NR dependent carcinomas. Hence screening of other adenocarcinomas, e.g. from the colon, endometrium or pancreas, would be an interesting next step. However, caution must be taken when addressing the prognostic value of a single marker, or a set of markers (gene expression signatures). Venet *et al.* (2011) recently demonstrated that most random signatures are significantly ($p<.05$ for 77%, and $p<10^{-5}$ for 30% of the tested signatures) associated with breast cancer outcome, without having a biological relation to cancer.[39] This is most likely caused by the fact that most genes of the breast carcinoma transcriptome are related to one variable, the proliferating cell nuclear antigen (PCNA). The PCNA gene encodes a protein that importantly regulates several processes leading to DNA replication. As a consequence, correcting for this variable abrogates most significant associations. Hence, the evaluation of markers against the outcome association of comparable negative control markers is highly important. Cell proliferation is an important factor in tumor biology, therefore deducing whether a change in the expression of a marker is a cause, or a consequence of increased cell proliferation, is crucial. Nonetheless, an accurate, but biological irrelevant marker may still be valuable in the clinic. The early loss of DC-SCRIPT expression in both breast- and prostate tumor cells makes it an interesting prognostic marker, however, it remains to be determined whether this is initiating, or following malignant transformation.

*NR balance and cell cycle regulation*

Whether DC-SCRIPT is a causative- or consequential marker, loss of expression very likely aids tumor growth and malignancy. In **chapter 5** we demonstrate DC-SCRIPT to co-repress the Type I NR ERα and PR-B, and enhance transcriptional activities of the Type II NR RARα/RXR and PPARγ.[6] In breast cancer cells, the activity of the NR plays an important role, as these NR affect both cell growth and apoptosis. Estrogens exert proliferative and pro-apoptotic effects in these cells, and are often seen as causative agents in breast cancer development.[33,40] In contrast, RARα/RXR and PPARγ are typically referred to as tumor suppressive, and counteract the effects of ERα in cell cycle control and apoptosis pathways.[41-43] In **chapter 6** we describe the opposing roles of ERα and RAR in the apoptosis pathway, cell cycle regulation and growth factor signalling.[33] Importantly, by regulating the activity of both NR types, DC-SCRIPT may balance their function in breast epithelial cells, preventing uncontrolled proliferation. A similar role for DC-SCRIPT is recognized in prostate carcinoma cells. Here, androgens drive cancer development, growth and survival, whereas VDR activation triggers anti-proliferative and differentiating effects.[44,45] DC-SCRIPT was shown to co-repress AR while enhancing VDR function, and may thereby function to restrict cell proliferation.[10]

Interestingly, the tumor-suppressive effect of DC-SCRIPT could also be demonstrated *in vitro*. In **chapter 5** DC-SCRIPT overexpression in MCF-7 breast carcinoma cells was shown to inhibit cell growth.[6] Recent studies with breast cancer cell lines Cama-1 and MDA-MB-231 (M. Ansems, personal communication) and the prostate carcinoma cell line 22Rv1[10] confirmed these results. In addition, expression profiling of ER-positive breast cancer tissue uncovered a negative association between high DC-SCRIPT and many cell cycle proteins. This is in-line with the transcriptional control of ERα on cell cycle-related genes, however, to date no direct effect of DC-SCRIPT expression on these ER-controlled cell cycle proteins could be discerned *in vitro*. In contrast, inducible expression of DC-SCRIPT in MCF-7 cells did observably affect cell cycle progression, with increased number of cells in the G1-phase, and less in the S-phase (M. Ansems, personal communication). Since ERα is known to induce G1- to S-phase transition, DC-SCRIPT possibly hampers ERα-mediated cell proliferation.[40,46,47]

These findings thus demonstrate an important role for DC-SCRIPT in both prostate and mammary adenocarcinomas, possibly as a tumor-suppressor gene. In the presence of functional NRs, DC-SCRIPT expression can control cell proliferation, hence the reduced or lost expression early in cancer development favors tumor growth. How DC-SCRIPT expression is regulated is currently unknown, and requires further research. One possible mechanisms is DNA methylation, however, studies with demethylation agents did not induce DC-SCRIPT expression. Other regulatory processes include histone modifications and transcription regulation via other TFs. Promoter studies and ChIP experiments for histone modifications and TF binding could increase insight into these issues. Data provided by the ENCODE (Encyclopedia of DNA Elements, http://genome.ucsc.edu/ENCODE/) consortium, which aims to identify all functional elements in the human genome, could be used to analyze the genomic site of DC-SCRIPT. This could provide detailed information regarding regulatory elements, epigenetic modifications and TF binding sites that regulate DC-SCRIPT expression. Ultimately, more detailed insight into the regulation of DC-SCRIPT expression and function may lead to more specific treatment strategies of these carcinomas.

## CONCLUSIONS

In this thesis DC-SCRIPT was demonstrated to have important regulatory roles on gene transcription in both DCs and epithelial cells. Although both cell types differ greatly in function and phenotype, overlapping roles of DC-SCRIPT on NR dependent transcription were detected. Cell specific effects were also recognized, such as regulation of IL-10 secretion in DC maturation and control of cell cycle progression in breast cancer cells. Future research is essential to provide a more detailed and more complete view on the function and regulation of DC-SCRIPT in both cell types. This can importantly aid understanding of DC- and tumor biology, and provide new insights for DC vaccinations and treatment strategies. It is, however, important to realize that transcription regulators such as DC-SCRIPT function in concert with other TFs and regulatory proteins, and that their activity therefore depends on the present molecular environment. DC-SCRIPT, and other TFs, should therefore not be seen as an individual acting proteins, but more as a members of a community, in which proteins tightly interact and influence each other's activity and function.

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# Chapter 8

## Nederlandse samenvatting

## SAMENVATTING VAN DIT PROEFSCHRIFT

Dendritische cellen zijn de centrale cellen van ons immuunsysteem die de afweer tegen infecties en tumoren regisseren. Het eiwit DC-SCRIPT komt voor in dendritische cellen en tumorcellen, en is essentieel voor het goed functioneren van de dendritische cel, terwijl DC-SCRIPT in de tumorcel de groei van de cel en van de tumor remt. In beide gevallen reguleert DC-SCRIPT de activiteit van belangrijke genen in deze cellen. Bovendien bepaald DC-SCRIPT de invloed van hormonen en vitaminen op de cellen, waarvan bekend is dat ze de groei en functie van de cel kunnen beïnvloeden**.**

## HET IMMUUNSYSTEEM

Het immuunsysteem, ook wel afweersysteem genoemd, beschermd ons, het menselijk lichaam, tegen bedreigingen van buitenaf (e.g. bacteriën en virussen), en van binnenuit (tumoren). Een belangrijke rol hierbij is weggelegd voor de dendritische cel, welke zowel de rol van verkenner, rechter en generaal vertolkt. Deze cellen vinden we overal in ons lichaam, waar ze met hun 'dendrieten' de omgeving afspeuren naar ongewenste indringers. Op deze manier worden ziekteverwekkers herkend, maar worden ook lichaamseigen cellen die tot tumorcel getransformeerd zijn opgespoord. Deze verdachte objecten worden door de dendritische cel opgenomen in een proces dat fagocytose wordt genoemd. Vanaf dit moment fungeert de dendritische cel als rechter, en beoordeeld of de opgenomen stoffen/cellen een gevaar vormen voor het lichaam. Is dat het geval, dan ondergaat de dendritische cel de transformatie naar generaal, en activeert het immuunsysteem. De dendritische cel presenteert delen van de opgenomen ziekteverwekker aan de effector cellen (soldaten) van het immuunsysteem, de T- en B-cellen. Ook bepaald de dendritische cel de strategie voor de aanval, door de T- en B-cellen te laten specialiseren in het opruimen van bacteriën, schimmels, virussen of tumoren. Op deze manier wordt de aanwezige ziekteverwekker op een zo efficiënt mogelijke manier bestreden.

In het zojuist beschreven proces past het immuunsysteem de afweerreactie aan op de ziekteverwekker. Deze vorm van afweer wordt daarom ook wel het adaptieve- of verworven immuunsysteem genoemd. Echter, naast de adaptieve immuniteit wordt ons lichaam ook beschermd door het aangeboren immuunsysteem. Dit systeem gaat, na herkenning van de ziekteverwekker, direct over in het ter plekke bestrijden van de infectie. Het is in feite de eerste verdedigingslinie van ons lichaam tegen externe ziekteverwekkers en tumoren, en kan de infectie bestrijden of controleren voordat het adaptieve immuunsysteem geactiveerd is.

Het aangeboren systeem bestaat uit een variëteit aan cellen (granulocyten, macrofagen, dendritisch cellen en monocyten) die door middel van speciale receptoren een groot scala aan ziekteverwekkers kunnen herkennen. Deze receptoren worden in het Engels ook wel Pattern Recognition Receptors (PRRs) genoemd, wat in feite betekend dat ze specifieke moleculaire patronen van bacteriën, virussen of schimmels kunnen herkennen. Recente ontwikkelingen in de wetenschap hebben aangetoond dat vooral de combinatie van verschillende PRRs belangrijk is voor de herkenning van een specifiek micro-organisme. **Hoofdstuk 2** beschrijft hoe deze receptoren door de cellen van het aangeboren immuunsysteem gecombineerd kunnen worden op moleculair niveau, en zo elkaars functie en activiteit beïnvloeden. Op deze manier kunnen de cellen van het aangeboren systeem niet alleen onderscheid kunnen maken tussen de verschillende klassen van micro-organismen, maar ook tussen verschillende soorten binnen dezelfde klasse.

## DC-SCRIPT EN DE DENDRITISCHE CEL

De verbindende factor tussen de twee afweersystemen is de dendritische cel. Deze cel behoord tot het aangeboren immuunsysteem, herkend ziekteverwekkers via zijn brede scala aan PRRs en activeert het adaptieve immuunsysteem. De dendritische cel presenteert delen van de ziekteverwekker aan het immuunsysteem, en produceert verschillende signaalstoffen, cytokinen genoemd. Deze cytokinen beinvloeden de activatie en specialisatie van T- en B cellen, zodat de immuunrespons aangepast wordt aan de aanwezige infectie. Zo is de productie van IL-12 belangrijk voor een optimale respons tegen virussen en tumoren, IL-4 tegen bacteriën en IL-23, TNF en IL-6 voor de bestrijding van schimmels. Tot slot kan de dendritische cel ook IL-10 uitscheiden, wat de cellen van het immuunsysteem remt, en zo voorkomt dat een immuunreactie wordt opgewekt tegen een ongevaarlijke stof of micro-organisme. In het laatste geval spreken we van tolerogene dendritische cellen, aangezien ze het immuunsysteem tolerant maken voor de aanwezigheid van een bepaalde stof of micro-organisme.

Het eiwit DC-SCRIPT speelt een belangrijke rol in deze processen, wat nader beschreven wordt in **hoofdstuk 3 en 4** van dit proefschrift. In **hoofdstuk 3** laten we zien dat DC-SCRIPT al vroeg in de ontwikkeling van de dendritische cel aanwezig is, en voornamelijk voorkomt in de kern van de cel, waar de genregulatie plaatsvindt. Als we de hoeveelheid DC-SCRIPT in de cel experimenteel verlagen, zien we dat de dendritische cel meer IL-10 en minder IL-12 gaan produceren, waardoor de activatie van T-cellen geremt wordt. De dendritische cel wordt dus meer tolerogeen in de afwezigheid van DC-SCRIPT.

Eenzelfde effect kan worden bewerkstelligd door de toevoeging van corticosteroïden. Dit stress-gerelateerde hormoon remt het immuunsysteem, en maakt dendritische cellen tolerant. Corticosteroiden worden om deze reden vaak toegepast in situaties waarbij het immuunsysteem geremd moet worden, zoals bij transplantaties, allergieën en auto-immuunziekten. Corticosteroïden activeren een receptor in de cel, de Glucocorticoid Receptor (GR), welke de activiteit van belangrijke genen reguleert. In **hoofdstuk 4** laten we zien dat DC-SCRIPT een remmende werking heeft op GR, en daarmee het effect dat het hormoon op de cel heeft reduceert. In de dendritische cel leidt de verlaging van DC-SCRIPT dan ook tot een verhoogde gevoeligheid voor corticosteroïden. Dit wijst erop dat DC-SCRIPT een belangrijke rol speelt in de transformatie die de dendritische cel ondergaat na detectie van ziekteverwekkers.

## DC-SCRIPT IN TUMORCELLEN

Behalve dendritische cellen hebben we ook aangetoond dat DC-SCRIPT een belangrijke rol speelt in de cellen van borsttumoren. Borsttumoren ontstaan uit de epitheel cellen die de klieren in de borst bekleden, en zijn vaak gevoelig voor het hormoon oestrogeen. Dit activeert de oestrogeen receptor (ER), die de activiteit van genen in de tumorcel reguleert, en de groei van de cellen en de tumor bevorderd. Patiënten worden om die reden vaak behandeld met anti-oestrogenen, die de werking van de oestrogeen receptor blokkeren. Echter, in sommige patiënten is de oestrogeen receptor niet meer aanwezig in de tumor, waardoor deze onafhankelijk geworden is van de effecten van oestrogeen. Behandeling met anti-oestrogenen is dan niet meer mogelijk.

**Hoofdstuk 5** laat zien dat DC-SCRIPT aanwezig is in gezonde epitheel cellen van het borstklierweefsel, maar afneemt zodra deze cellen transformeren naar tumorcellen. Deze afname gaat gepaard met een verhoogd risico op terugkeer van de tumor na de operatieve verwijdering. Patiënten waarbij weinig tot geen DC-SCRIPT in de borsttumor aanwezig was hadden een hogere kans op terugkeer van de ziekte vergeleken met patiënten waar relatief hoge hoeveelheden DC-SCRIPT in de tumor gevonden werden. Echter, dit effect is alleen aantoonbaar bij oestrogeen gevoelige tumoren. In patiënten met een oestrogeen ongevoelige tumor had de aanwezigheid van DC-SCRIPT geen voorspellende waarde.

Om dit effect nader te verklaren hebben we de effecten van DC-SCRIPT op de werking van de oestrogeen receptor onderzocht. **Hoofdstuk 5** laat zien dat de aanwezigheid van DC-SCRIPT in een cel de activiteit van de oestrogeen receptor remt. Bovendien blijkt dat DC-SCRIPT een stimulerende werking heeft op de effecten van o.a. vitamine A. In tegenstelling tot oestogeen, is vitamine A voornamelijk bekend om zijn remmende effect op de groei van borsttumoren. Doordat DC-SCRIPT de werking van vitamine A versterkt, terwijl het de effecten van oestrogeen remt, kan het een belangrijke factor zijn in het voorkomen borstkanker.

Van de werking van de oestrogeen- en vitamine A receptoren in borsttumoren is veel bekent. Onderzoek heeft aangetoond dat deze receptoren onafhankelijk van elkaar verschillende genen reguleren, maar ook samenwerken, of elkaar tegenwerken bij de regulatie van andere genen. Deze zogenaamde ‘crosstalk’, en de rol hiervan in tumorcellen, wordt besproken in **hoofdstuk 6**. Aangezien vaak meerdere hormonen en vitaminen in de omgeving van de tumor aanwezig zijn, is het interessant om onderzoeken wat de effecten van deze crosstalk op de cellen zijn, en hoe DC-SCRIPT daarbij betrokken is.

## TOEKOMSTPERSPECTIEVEN

In dit proefschrift hebben we laten zien dat DC-SCRIPT een belangrijke rol speelt in zowel dendritische cellen als tumorcellen. In beide gevallen reguleert DC-SCRIPT de activiteit van belangrijke genen, en beïnvloed het de effecten die hormonen en vitaminen op deze cellen hebben. Echter, hoe dit op moleculair niveau gebeurt is nog grotendeels onbekend. Er ligt daarom een grote uitdaging om de onderliggende mechanismen te ontdekken. Details over de precieze werking en regulatie van DC-SCRIPT in deze cellen kan potentieel nieuwe inzichten in de biologie van dendritische cellen en tumorcellen verschaffen. Bovendien zou deze kennis in de toekomst waardevol kunnen zijn voor het ontwikkelen van op dendritische cel gebaseerde immuun therapieën en kankerbestrijding.

# Chapter 9

## Epilogue

*Dankwoord*

*Curriculum Vitea*

*List of publications*

*List of acronyms*

## DANKWOORD

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*Saartje*

## CURRICULUM VITEA

*Saartje Hontelez* werd geboren op 31 mei 1983 te Wageningen. Na het behalen van haar VWO diploma aan het Pantarijn te Wageningen, begon zij in september 2001 aan de studie Biologie aan de Wageningen Universiteit. In 2005 behaalde ze hiervoor haar Bachelors diploma. Tijdens de daaropvolgende Masterfase heeft zij, bij dezelfde universiteit, een tweetal onderzoeksstages van 6 maanden voltooid. De eerste stage betrof een onderzoek naar de moleculaire achtergrond van het leergedrag van sluipwespen, en werd uitgevoerd bij de vakgroep Entomologie, onder begeleiding van Dr. Hans M. Smid. De tweede stage was gericht op de fenotypische karakterisering van een *Arabidopsis thaliana* mutant, welke deficiënt is voor een eiwit van het exocytose complex, en werd, onder begeleiding van Dr. Tijs Ketelaar, uitgevoerd bij de vakgroep Plant Cel Biologie. Ze sloot haar studie af met een buitenlandse stage aan de Universiteit van New South Wales te Sydney, waar ze bij de vakgroep van Prof. Stephen J. Simpson onderzoek deed naar het foerageergedrag van de plaagsprinkhaan *Locusta migratoria*. In 2007 behaalde ze haar Masters diploma.

Aansluitend begon zij als onderzoeker in opleiding (OIO) aan haar promotietraject bij de afdeling Tumor Immunologie aan het UMC St. Radboud, onder begeleiding van Prof. Gosse J. Adema. Tijdens deze periode werd het in dit proefschrift beschreven promotieonderzoek uitgevoerd. Vanaf januari 2012 is zij werkzaam als post-doc bij de vakgroep Molecular Developmental Biology aan de Radboud Universiteit, onder begeleiding van Prof. Gert Jan Veenstra, waar zij onderzoek doet naar de rol en oorsprong van de epigenetica in de vroege embryonale ontwikkeling.

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*Authors contributed equally

# LIST OF ACRONYMS

| | |
|---|---|
| AF-1 | Activation Function 1 |
| Ag | Antigen |
| AIB | Amplified in Breast Cancer |
| AP-1 | Acivator Protein 1 |
| APC | Antigen Presenting Cells |
| AR | Androgen Receptor |
| AtRA | All trans Retinoic Acid |
| B-cell | B lymphocytes |
| Bcl | B-cell Cll/lymphoma |
| BCR | B-cell receptor |
| BDCA | Blood Dendritic Cell Antigen |
| CARD | Caspase Recruitment Domain family |
| CCN | Cell Cyclin |
| CCR | chemokine (C-C motif) receptor |
| CD | Cluster of Differentiation |
| CDP | Common DC Precursor |
| ChIP | Chromatin Immunoprecipitation |
| ChIP-seq | ChIP-sequencing |
| CI | Confidence Interval |
| CLR | C-type Lectin Receptor |
| CLSM | Confocal Laser Scanning Microscopy |
| Co-IP | Co- Immunoprecipitation |
| CR | Complement Receptor |
| CRD | Carbohydrate Recognition Domain |
| CREB | cAMP Responsive Element Binding protein |
| CtBP | C-terminal Binding Protein |
| CTL | Cytotoxic T-cell |
| DAMP | Danger Associated Molecular Pattern |
| DBD | DNA Binding Domain |
| DC | Dendritic Cell |
| DCIR | Dendritic Cell Immunoreceptor |
| DC-SCRIPT | Dendritic Cell-specific tranSCRIPT |
| DC-SIGN | Dendritic Cell-Specific Intracellular adhesion molecules-3 Grabbing Non-integrin |
| Dex | Dexamethasone |
| DUSP | Dual Specific Phosphatase |
| ECM | Extracellular Matrix |
| EGFR | Epidermal Growth Factor Receptor |
| ELISA | Enzyme-linked immunosorbent assay |
| ER | Estrogen Receptor |
| ERE | ER Responsive Element |
| ERK | Extracellular signal-Regulated Kinase |
| ERR | Estrogen Related Receptor |
| FcR | Fc Receptor |
| FDC | Follicular DC |
| FKBP5 | FK506 Binding Protein |

| | |
|---|---|
| FoxA1 | Forkhead box P3 |
| GC | Glucocorticoid |
| Gfi | Growth factor independence |
| GFP | Green Fluorescent Protein |
| GILZ | GC Inducible Leucine Zipper |
| GM-CSF | Granulocyte Macrophage Colony Stimulating Factor |
| GR | Glucocorticoid Receptor |
| GRE | GR Responsive Element |
| HAT | Histone Acetyl Transferase |
| HDAC | Histone Deacetylase |
| HEK293 | Human Embryonic Kidney 293 |
| HEP3B | Human hepatocellular carcinoma 3B |
| HR | Hazard Ratio |
| HRE | Hormone Responsive Element |
| iDC | immature DC |
| IFN | Interferon |
| IGF | Insuline-like Growth Factor |
| IGF-1R | Insuline-like Growth Factor 1 Receptor |
| Ig | Immunoglobulin |
| IHC | Immunohistochemistry |
| IL | Interleukin |
| IL-1R | Interleukin-1 Receptor |
| IP | Immunoprecipitation |
| IRF | Interferon Regulatory Factor |
| ITAM | Immunoreceptor Tyrosine-based Activation Motif |
| JNK | JUN N-terminal kinase |
| LBD | Ligand Binding Domain |
| LC | Langerhans Cells |
| LP | Lymphoid Precursors |
| LPS | Lipopolysaccharide |
| LRR | Leucine Rich Region |
| LXR | Liver X Receptor |
| MAPK | mitogen-activated protein kinase |
| MCF-7 | Michigan Cancer Foundation - 7, human breast cancer cell line |
| mDC | mature DC |
| MDC | Myeloid DC |
| MHC | Major Histocompatibility Complex |
| MKP | Mitogen-activated protein Kinase Phosphatase |
| MLR | Mixed Leukocyte Reaction |
| MMTV | Mouse Mammary Tumor Virus |
| moDC | monocyte derived DC |
| MP | Myeloid Precursor |
| MR | Mannose Receptor (chapter 2) or Mineralcorticoid Receptor (chapter 7) |
| MyD88 | Myeloid Differentiation primary response gene (88) |
| N/C ratio | Nuclear/Cytoplasmic ratio |
| NCOA | Nuclear receptor Coactivator |

| | |
|---|---|
| NCoR | Nuclear receptor Corepressor |
| NF1 | Nuclear Factor 1 |
| NFAT | Nuclear Factor of Activated T-cells |
| NF-κB | Nuclear Factor-κB |
| nGRE | negative GRE |
| NK cell | Natural Killer cell |
| NLR | NOD-like Receptor |
| NLRP | NLR family, pyrin domain containing |
| NLS | Nuclear Localization Signal |
| NR | Nuclear Receptor |
| Oct1 | Octamer Transcription Factor 1 |
| ORF | Open Reading Frame |
| PAMP | Pathogen Associated Molecular Patterns |
| PBGD | Porphobilinogen Deaminase |
| PBL | Peripheral blood lymphocyte |
| PBMC | Peripheral blood mononuclear cell |
| PCNA | Proliferating Cell Nuclear Antigen |
| PCR | Polymerase Chain Reaction |
| PDC/pDC | Plasmacytoid DC |
| poly(I:C) | Polyinosinic:polycytidylic acid |
| PPAR | Peroxisome Proliferator-Activated receptor |
| PR | Progesterone Receptor |
| Pred | Prednisolone |
| preDC | precursor DC |
| PRR | Pattern Recognition Receptor |
| PTM | Post Translational Modification |
| PTX3 | Pentraxin 3 |
| R848 | Resiquimod |
| RA | Retinoic Acid |
| RAR | Retinoic Acid Receptor |
| RARE | RAR Responsive Element |
| rh-DC-SCRIPT | recombinant human DC-SCRIPT |
| RIP140 | Receptor Interacting Protein 140 |
| RLR | Retinoic acid-inducible gene (RIG)-I like receptors |
| ROR | Retinoid acid-related receptor |
| ROS | Reactive Oxygen Species |
| RU-486 | Mifepristone |
| RXR | Retinoic X Receptor |
| siIL-10 | IL-10 targeting siRNA |
| siRNA | small interfering RNA |
| siSC | DC-SCRIPT targeting siRNA |
| SL2 | Schneider Drosophila Line 2 |
| SMRT | Silencing Mediator for Retinoid and Thyroid hormone receptors |
| SRC | Steroid Receptor Coactivator |
| STAT | Signal Transducer and Activator of Transcription |
| SWI/SNF | Switch/Sucrose Nonfermentable |

| | |
|---|---|
| T-cell | T lymphocyte |
| TCR | T-cell Receptor |
| TF | Transcription Factor |
| TFF | Trefoil Factor |
| TGF | Transforming Growth Factor |
| Th-cell | T helper cell |
| THP-1 | Human acute monocytic leukemia cell line |
| TLR | Toll-Like Receptor |
| TNF | Tumor Necrosis Factor |
| tolDC | tolerogenic DC |
| TR | Thyroid hormone Receptor |
| Treg | regulatory T-cell |
| VDR | Vitamin D3 Receptor |
| YFP | Yellow Fluorescent Protein |
| ZNF366 | Zincfinger protein 366 |